Role of prostaglandins in early CL formation in the dog: effects on vascularization, immunoactive factors and global transcriptomic changes

Inaugural Thesis

to obtain the title of Doctor of Veterinary Medicine and Philosophy
(Dr. sc. med. vet.; corresponds to DVM, PhD)

from the Vetsuisse Faculty University of Zurich

submitted by

Miguel Augusto Tavares Pereira

approved by

Prof. Dr. Mariusz P. Kowalewski, PhD (Supervisor)

Prof. Dr. Tomasz Janowski (Co-advisor)

Prof. Dr. Torsten Seuberlich (Mentor)

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Abbreviations

3βHSD – 3-beta-hydroxysteroid dehydrogenase
ANGPT (1/2) – angiopoietin (1/2)
ANOVA – analysis of variance
AP1 – activator protein 1
cAMP – cyclic adenosine monophosphate
CCL (3/13) – C-C motif chemokine ligand (3/13)
CCNA2 – cyclin A2
CD (4/8) – cluster of differentiation 4/8)
cJUN – JUN proto-oncogene
CL – corpus luteum
dbcAMP – N6,2-dibutyryladenosine-3,5-cyclic monophosphate
DEGs – differentially expressed genes
e.g. – *exempli gratia*, for example
E1 – estrone
E2 – 17β-estradiol
ECE1 – endothelin converting enzyme 1
ECM – extracellular matrix
EIF4H – eukaryotic translation initiation factor 4H
END – endoglin
ERα/ESR1 – estrogen receptor alpha
ERβ/ESR2 – estrogen receptor beta
ET (1-3) – endothelin (1-3)
ET (A/B) – endothelin receptor (A/B)
FAS – Fas cell surface death receptor
FASLG – Fas ligand
FGF (1/2) – fibroblast growth factor (1/2)
FSH – follicle stimulating hormone
GATA (4/6) – GATA binding protein (4/6)
GLUT1/SLC2A1 – glucose transporter 1/solute carrier family 2 member 1
GnRH – gonadotropin-releasing hormone
HSD17B7 – hydroxysteroid 17b dehydrogenase 7
i.a. – *inter alia*, among other things
i.e. – *id est*, that is
ICAM1 – intercellular adhesion molecule 1
IHC – immunohistochemistry
IL (1β/6/8/10/12a) – interleukin (1 beta/6/8/10/12 subunit alpha)
IP3 – inositol 1,4,5-triphosphate
KDM4A – lysine (K)-specific demethylase 4A
LH – luteinizing hormone
MHCII – major histocompatibility complex II
NFκB (1) – nuclear factor kappa B (subunit 1)
NFκBIA – NFKB inhibitor alpha
NGS – next generation sequencing
NODAL – Nodal growth differentiation factor
OHE – ovariohysterectomy
p.o. – post-ovulation
P4 – progesterone
PG – prostaglandin
PGD2 – prostaglandin D2
PGE2 – prostaglandin E2
PGF2α – prostaglandin F2α
PGG2 – prostaglandin G2
PGH2 – prostaglandin H2
PGI2 – prostaglandin I2
PGR – nuclear progesterone receptor
PPARγ – peroxisome proliferator activated receptor gamma
PTGDR/DP – prostaglandin D receptor
PTGDS – prostaglandin D synthase
PTGER (1-4)/EP (1-4) – prostaglandin E receptor (1-4)
PTGES – prostaglandin E synthase
PTGFR/FP – prostaglandin F receptor
PTGFS – prostaglandin F synthase
PTGIR/IP – prostaglandin I receptor
PTGIS – prostaglandin I synthase
PTGS/COX (1/2) – prostaglandin synthase / cyclooxygenase (1/2)
PTK2 – protein tyrosine kinase 2
RNA-Seq – RNA sequencing
RT-qPCR – semi quantitative real time (TaqMan) polymerase chain reaction
SD – standard deviation
SF1 – steroid factor 1
STAR – steroidogenic acute regulatory protein
STS – steroid sulfatase
SULT1E1 – sulfotransferase family 1E member 1
TXA2 – thromboxane A2
TBXAS1 – thromboxane A synthase 1
TBXAS2R/TP – thromboxane A2 receptor
TGFβ – transforming growth factor β
THBS (1) – thrombospondin (1)
Tie (1/2) – tyrosine kinase with immunoglobulin-like and EGF-like domains (1/2)
TNFR (1/2) – tumor necrosis factor receptor (1/2)
TNFα – tumor necrosis factor alpha
VEGF (A) – vascular endothelial growth factor (A)
VEGFR – vascular endothelial growth factor receptor
Xg – geometric mean
YY1 – transcription factor Ying-Yang 1

Abstract

The reproductive cycle of the dog (*Canis lupus familiaris*) presents a plethora of unique features when compared with other mammals. The absence of steroidogenic activity in the placenta gives the *corpus luteum* (CL) a crucial role in the outcome of pregnancy. Furthermore, the lack of an active luteolytic principle in the form of extraluteal (i.e., uterine) or intraluteal prostaglandin (PG) F2α in non-pregnant bitches results in the presence a prolonged physiological pseudopregnancy. The latter is frequently longer than pregnancy itself, presenting circulatory progesterone (P4) levels similar to those of pregnant animals, and terminates with a slow and passive CL degeneration. Adding to these events the long obligatory sexual quiescence (anestrus), an extended reproductive cycle is observed in the dog, in which the CL plays a central role in its regulation. Thus, understanding of CL regulation and function is critical for the understanding of canine reproduction.

The regulation of CL function in the dog is also peculiar. It can be subdivided into two regulatory stages: an early gonadotropin-independent period of CL formation and development, and the mature CL dependent on gonadotropins, predominantly prolactin (PRL), for its function. The early independence on hypophysial hormones suggests an intrinsic regulation of the CL through factors acting in an auto/paracrine way. Prostaglandins (PGs), primarily PGE2, are proposed to be among these factors. Prostaglandins are active lipid compounds that exhibit hormone-like effects in different systems. In the reproductive cycle, functional responsibilities between the luteotropic PGE2 and luteolytic PGF2α were demonstrated in several species. In the dog, the idea that PGE2 could perform luteotropic roles derived from the observed increased availability of PTGS2/COX2 and PGE2 synthase (PTGES) that led to increased levels of PGE2 within the CL during its early stages. In addition, the stimulatory role of PGE2 on steroidogenesis in primary canine luteal cells supports this idea. Furthermore, the *in vivo* effects of firocoxib (Previcox®, Merial), a COX2-specific inhibitor, provided the final demonstration of the luteotropic effects of PGs in the dog. In this study, non-pregnant bitches treated for up to 30 days after ovulation with firocoxib exhibited lower luteal levels of PGE2, lower expression of several steroidogenic factors and, consequently, lower circulating levels of P4. Besides these direct effects of PGs, predominantly of PGE2 in steroidogenesis, further indirect effects were observed *in vitro*, where PGE2 regulated expression of the vasodilatory endothelin (ET) receptor B (ETB) and ET2, and of angiopoietin 1 and -2 (ANGPT1, -2) which are involved in vascular stabilization. Further indirect effects of PGs appear to involve regulation of CL sensitivity to hormones, such as PRL, by affecting the expression of its receptor (PRLR).

In this context, the goal of the present PhD work was to extend knowledge on the modulatory role of PGs in the CL. For this, samples from the previously described *in vivo* study were used. These samples were divided into control and treated groups (n=3-5 animals/group), collected on the day of ovulation (day 0) or at days 5, 10, 20 or 30 after ovulation.

The previously described effects of PGE2 on vascular factors, and its regulatory effects on the activity of the immune system in several organs, prompted the hypothesis that PGs could be involved in the regulation of CL vascularization and immune response. In fact, based on the results obtained in this PhD thesis, PGs appear to exhibit both vaso- and immunomodulatory effects in the canine CL. After treatment, the expression of vascular factors belonging to the ANGPT-system, evaluated with semi-quantitative real time (TaqMan) qPCR, was decreased. In addition, the expression of endothelin 1 (ET1; typically linked to luteolysis), of pro-inflammatory interleukins (IL) -1β, -6 and -12, and of CD4, was increased ($P>0.05$) after treatment. Immunohistochemistry was used to validate these effects at the protein level. Interestingly, these Previcox-evoked changes related mostly to days 20 and 30.

The effects observed in the first study upon withdrawal of PGs encouraged us to perform a deep RNA sequencing (RNA-Seq; Next Generation Sequencing, NGS). Treatment-dependent effects were assessed by comparing samples from treated and control dogs. Interestingly, these effects appeared to be stage-dependent, reflected in large differences in the number of differentially expressed genes (DEGs, $P<0.01$, $FDR<0.1$) per contrast, and absence of genes concomitantly affected in all studied time-points. Days 5 and 10 (representing the early gonadotropin-independent stage) were weakly affected by treatment, presenting respectively 74 and 2 DEGs. These low effects in the early developing CL suggest the presence of compensatory mechanisms to treatment. In contrast, higher numbers of DEGs (1741) were found on day 20 (representing the transition towards gonadotropin-dependency), mainly related to increased cellular growth/proliferation and immune function. The 552 DEGs found on day 30 (representing the mature PRL-dependent CL) were mainly related to decreased steroidogenic function and vascularization. This analysis suggested the presence of stage-dependent and multidirectional effects of COX2-inhibition in luteal function and the presence of strong compensatory effects in the early canine CL. In addition to treatment-induced effects, time-dependent changes in the expression of many factors were also analyzed. Several of these were described for the first time in the CL, being involved in luteal vascularization, immunity, sensitivity to steroids and transcriptional regulation of STAR.

Cumulatively, this PhD work provides new information regarding the effects of PGs in the modulation of CL function and changes in mechanisms regulating maturation of the CL. The translational value of the study for other domestic animal species needs to be emphasized.

1. Introduction

1.1 Prostaglandins

1.1.1. Synthesis, signaling and main roles

Prostaglandins (PGs) are arachidonic acid-derived eicosanoids (active lipid compounds) exhibiting hormone-like effects in the body. The family includes PGE2, PGF2α, PGI2, PGD2 and thromboxane A2 (TXA2). These lipid compounds are found in most tissues, acting in a plethora of mechanisms, including inflammation, vascular relaxation and/or constriction, platelet aggregation, sleep regulation, bronchoconstriction, allergic reactions and regulation of reproduction (Breyer, et al. 2001, Hata and Breyer 2004, Murdoch, et al. 1993, Weems, et al. 2006). Due to their short life period, PGs act mostly at a local level through their specific transmembrane receptors. Specific receptors were described for particular prostaglandins [(Fig.1) (Breyer, et al. 2001, Hata and Breyer 2004)]. Additionally, splice variants could be observed in some cases, e.g., for PTGFR/FP, TBXA2R/TP, PTGER1/EP1 and PTGER3/EP3 (Breyer, et al. 2001). Regarding PGE2, four receptor subtypes were described: PTGER1/EP1, -2, -3 and -4 [(Fig.1) (Breyer, et al. 2001, Hata and Breyer 2004)]. Their activity varies based on structure, but also on their affinity to the ligand (PGE2) and the downstream pathways they activate. For example, while activation of PTGER1/EP1 leads to inositol 1,4,5-triphosphate (IP3)-dependent increase in intracellular calcium ion concentration, PTGER2/EP2 and -4 signals are cAMP-mediated (Breyer, et al. 2001, Hata and Breyer 2004).

The activity of prostaglandin-endoperoxide synthases/cyclooxygenases (PTGS1/COX1 or PTGS2/COX2) is indispensable in PGs biosynthesis (Smith 1992, Smith and Dewitt 1996). Of these, PTGS2/COX2 is an inducible enzyme that is expressed in response to different stimuli such as cytokines and growth factors (Hata and Breyer 2004, Smith and Dewitt 1996). These enzymes exhibit both dioxygenase and peroxidase activity, converting membrane-derived arachidonic acid (released by phospholipase A2), via PGG2 as an intermediate, into PGH2 [(Fig. 1) (Smith 1992, Smith and Dewitt 1996)]. In subsequent downstream synthesis steps, PGH2 functions as a single precursor for all active PGs [(Fig.1) (Smith 1992)]. Moreover, some enzymes can interconvert PGs, as in the case of PGE2-9-ketoreductase which utilizes PGE2 for PGF2α synthesis (Watson, et al. 1979). Such a mechanism has been implicated as the main PGF2α synthesis route in the canine prepartum placenta (Gram, et al. 2014). Thus, the production of different PGs in specific tissues is determined by the expression of their synthases and/or converting enzymes (Breyer, et al. 2001, Smith 1992). Notably, the inhibition of PGs synthesis can be achieved by blocking the activity of COX1 and/or COX2 using different compounds that might present a higher affinity/specificity for one of these enzymes. In this way, e.g., acetylsalicylic acid inhibits COX1 activity, oxicams (e.g.,

meloxicam and piroxicam) are preferential inhibitors of COX2 but also affect COX1 activity, coxibs (e.g., celecoxib, robenacoxib and firocoxib) are COX2-specific inhibitors while indomethacin is a nonselective inhibitor of both cyclooxygenases (FitzGerald and Patrono 2001, Smith and Dewitt 1996, Weems, et al. 2006).

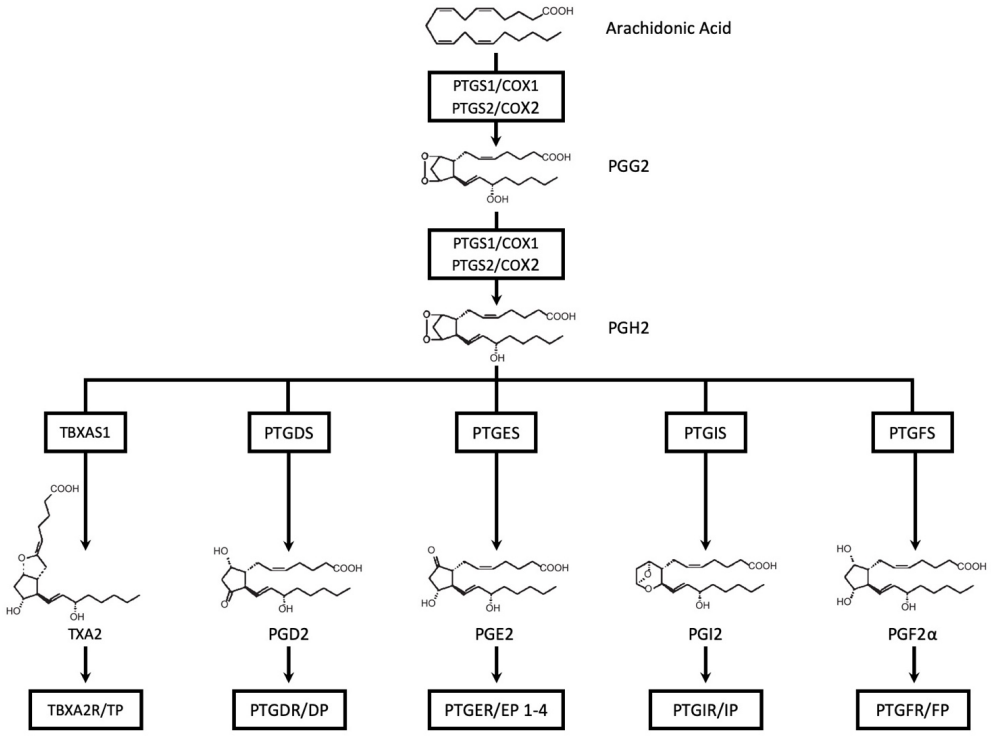

Figure 1. Prostaglandin synthesis pathway. Arachidonic acid is converted into PGG2 and then into PGH2 by PTGS/COX1 and -2. PGH2 can then be converted into TXA2, PGD2, PGE2, PGI2 or PGF2α through their specific synthases which bind to PG-specific receptors (Adapted from Hata and Breyer 2004).

1.1.2. Roles in female reproduction

In female reproductive physiology, the effects of PGE2 and PGF2α are considered indispensable in several mechanisms. Both PGE2 and PGF2α have been found in follicular fluid and modulate oocyte maturation (Murdoch, et al. 1993, Weems, et al. 2006). For ovulation to occur, PGF2α locally induces collagenolysis in the Graafian follicle and the use of COX-blockers can affect or even prevent ovulation in species such as the rat, mouse, cattle, pig, horse, sheep or human (reviewed in Murdoch, et al. 1993). Also, horse embryos secrete PGE2 before implantation (Weber, et al. 1991), while in the dog and pig an increased expression of endometrial PTGES is observed in the presence of embryos (Kautz, et al. 2014, Wasielak, et al. 2009). In the murine uterus, PGE2 also exhibits immuno-modulatory effects that implicate its support in the feto-maternal immune tolerance (Mason, et al. 2013). Interestingly, PGE2 and PGF2α frequently exhibit opposing effects. For example, in the uterus, PGF2α induces smooth muscle contraction, either directly or by stimulating increased 17β-estradiol (E2) levels, while PGE2 inhibits it (Murdoch, et al. 1993, Weems, et al. 2006). These apparently antagonistic roles of both PGs are highlighted through their

roles in regulating *corpus luteum* (CL) function. Thus, whereas PGE2 exhibits luteotropic effects, PGF2α is the strongest known luteolytic insult (Niswender, et al. 2000, Pharriss and Wyngarden 1969, Weems, et al. 2006). Being mainly produced in the uterus, for transport of PGF2α to the ovary a transit through a counter-current mechanism from the utero-ovarian vein to the ovarian artery has been proposed (Ginther 1974). In addition, administration of PGF2α can also shorten postpartum intervals and is routinely used in reproductive cycle synchronization in livestock (Islam 2011, Weems, et al. 2006). Contrasting with these effects, in species like the dog, cattle and sheep, PGE2 stimulates luteal production of progesterone (P4), which is essential for the maintenance of pregnancy (Kim, et al. 2001, Kowalewski, et al. 2013, Niswender, et al. 2000, Stouffer and Hennebold 2015, Weems, et al. 1998). Thus, based on the available literature and empirical applications, it appears clear that the balance between PGE2- and PGF2α-driven effects in the CL is crucial for regulation of the female reproductive cycle.

1.2. Morpho-functional aspects of luteal formation and function

Progesterone is the dominant steroid of diestrus. It is crucial for the regulation of female reproduction. For its synthesis, cholesterol is converted by cytochrome P450 side-chain cleavage (P450scc; located at the inner mitochondrial membrane) into pregnenolone, which is then converted by 3β-hydroxysteroid dehydrogenase (3βHSD; found in the endoplasmic reticulum) into P4 (Stocco 2001). The rate-limiting step in the steroidogenic pathway is the transfer of cholesterol from the outer to the inner mitochondrial membrane in order to provide P450scc with the substrate. This step is mediated by the steroidogenic acute regulatory protein [(STAR) (Stocco 2001)]. The main targets of P4 during diestrus are the ovary itself (CL), the hypothalamus-hypophysis axis and the uterus (reviewed in Niswender, et al. 2000). In the hypothalamus-hypophysis axis, P4 blocks gonadotropin-releasing hormone (GnRH) signaling, by decreasing hypothalamic release of this hormone and hypophysial sensitivity to it, thereby decreasing the production of luteinizing hormone (LH) by the adenohypophysis (reviewed in Niswender, et al. 2000). In this way, P4 regulates, at least in part, the length of the reproductive cycle. In the uterus, this hormone is implicated in the modulation of endometrial protein expression, induction of stromal differentiation, stimulation of glandular secretion and inhibition of myometrial contractions (reviewed in Niswender, et al. 2000). For these reasons, a constant supply of P4 is indispensable for the successful outcome of pregnancy. Initially, in all mammals, the source of P4 is the CL which can be later replaced in some species by other sources such as the placenta, e.g., in humans or sheep (Stouffer and Hennebold 2015). In other species, like dogs, the constant provision of luteal P4 is essential for the success of pregnancy (Concannon, et al. 1989).

The CL is a highly complex tissue, composed of steroidogenic cells, endothelial cells, pericytes, fibroblasts and immune cells (Fraser and Wulff 2003). CL formation is a singular event in adult mammals, exhibiting intense proliferative, differentiative and tissue remodeling processes (Stouffer and Hennebold 2015). Following the preovulatory LH surge and ovulation, the CL is formed from the remaining follicular cells. Nevertheless, the process of luteinization commences prior to follicular rupture, involving both mural granulosa and *theca interna* cells (Niswender, et al. 2000). Then, following ovulation, the follicular antrum collapses and the basal membrane is disrupted, with *theca* cells being dislocated into the follicular cavity and differentiating into luteal cells (Stouffer and Hennebold 2015). In most domestic mammals, two populations of luteal cells can be discerned: large luteal cells, derived predominantly from the granulosa layer, and small luteal cells, derived from the *theca interna* layer (Corner 1919). However, this situation does not apply to the domestic dog, in which only one type of luteal cell can be observed morphologically and functionally (Hoffmann, et al. 2004b, Kowalewski, et al. 2015).

As with other endocrine glands, the CL requires a large blood supply. For this reason, the CL contains a dense vascular network that allows, directly or through the interstitial space, access of all luteal cells to blood vessels (Martelli, et al. 2009). Therefore, at the time of ovulation, the breakdown of the basal membrane allows blood vessels to spread into the follicular cavity, accompanied by extravasation of blood (Fraser and Wulff 2003, Stouffer and Hennebold 2015). This is continued in the form of intensive angiogenesis (Fraser and Wulff 2003). Several factors are involved in this process, including vascular endothelial growth factor (VEGF) family members and angiopoietin (ANGPT) system members (Fraser and Wulff 2003, Stouffer and Hennebold 2015). Among the most potent angiogenic factors stimulating endothelial cell proliferation, migration and tube formation, is VEGF (Ferrara and Davis-Smyth 1997). Acting through its receptors -1 and -2 (VEGFR1 and VEGFR2) it enhances vascular permeability, allowing vascular sprouting (Ferrara and Davis-Smyth 1997, Fraser and Wulff 2003, Kamat, et al. 1995). VEGFA expression is regulated by LH, indicating the modulatory capacity of hormones over luteal vascularization (Fraser and Wulff 2003, Hazzard and Stouffer 2000). In addition, other non-mitogenic vascular factors, like angiopoietins (ANGPTs), are also important for vascular development. Both ANGPT1 and -2, as well as their tyrosine kinase receptors Tie1 and -2, are present in the CL, localized in different cellular compartments, including luteal, endothelial and immune cells (Fraser and Wulff 2003, Gram, et al. 2018, Stouffer and Hennebold 2015). ANGPT1 acts as a stabilizer of blood vessels through the recruitment of pericytes, while ANGPT2 loosens them (Brindle, et al. 2006, Hanahan 1997, Hazzard and Stouffer 2000). In the absence of other angiogenic factors, ANGPT2 destabilizes blood vessels, leading to their degeneration (Felcht, et al. 2012, Hazzard and Stouffer 2000, Maisonpierre, et al. 1997). However, in the presence of VEGF, the ANGPT2-induced

disaggregation fosters the proliferation of endothelial cells (Felcht, et al. 2012, Hanahan 1997, Yancopoulos, et al. 2000). Accordingly, the ratio ANGPT1:ANGPT2, as well as the interaction between the VEGF- and ANGPT systems, appear to be essential in the development and maintenance of luteal vascularization. Complementing the functions of VEGFs and ANGPTs in regulating luteal function, endothelins (ETs) are involved in the regulation of vascular contractility (Schneider, et al. 2007, Yanagisawa, et al. 1988, Yanagisawa and Masaki 1989). Their roles in regulating the CL relate to processes of vascularization, steroidogenesis and arrest of luteal activity (Apa, et al. 1998, Meidan and Levy 2007).

The population of immune cells in the CL varies greatly throughout its life span (Hoffmann, et al. 2004a, Nowaczyk, et al. 2017, Pate and Keyes 2001, Stouffer and Hennebold 2015, Walusimbi and Pate 2013). In fact, both ovulation and luteolysis are considered inflammatory processes (Duffy, et al. 2019, Espey, et al. 2004, Niswender, et al. 2000, Pate and Keyes 2001, Stouffer and Hennebold 2015, Walusimbi and Pate 2013). During luteolysis a recruitment of immune cells to the CL and increased production of cytokines and other immune modulators is observed in several species, e.g., primates, dogs, ruminants, pigs and rodents (Nowaczyk, et al. 2017, Pate and Keyes 2001, Stouffer and Hennebold 2015, Walusimbi and Pate 2013). The immune system is involved in phagocytosis of degenerated cells, stimulation of luteal PGF2α secretion and inhibition of steroidogenic activity (reviewed in Niswender, et al. 2000). On the other hand, it is also involved in luteal development and function. Thus, e.g., in mice, depletion of macrophages disrupts the angiogenic process and, thereby, luteal integrity (Care, et al. 2013, Turner, et al. 2011). In exerting their actions, immune cells interact with and modulate the function of different luteal cell populations. However, the full extent of roles of the immune system in regulating the CL, as well as immune cell recruitment and interaction with other luteal cells, is still not well understood.

Despite the important role of the CL in female reproduction, knowledge regarding its regulation and function is still incomplete. The complexity and temporary nature of this organ present several obstacles to its research. In fact, the CL is composed of several cellular populations that may present stage-dependent (i.e., luteal development, maintenance or regression) variations in their presence and/or function. Finally, the presence of several luteotropic factors, like gonadotropins (FSH and LH), PRL, PGs and P4, and their different species-specific effects, also limits the translation of knowledge among different species.

1.3. Species-specific features of canine reproduction

1.3.1. Overview of the canine reproductive cycle and main endocrinological features

When compared with other mammals, the canine reproductive cycle exhibits several unique characteristics regarding its duration and main regulatory mechanisms. The main endocrine events in the canine reproductive cycle are shown in Fig. 2.

Being monoestric and generally a non-seasonal breeder, the bitch has a relatively long reproductive cycle when compared with other domestic mammals (Concannon 2011, Kowalewski 2018). One of the main contributors to this cycle length is the presence of a long and obligatory anestrus period (Concannon 2011, Kowalewski 2018).

A characteristic feature of the canine reproductive cycle is the strong preovulatory luteinization, which already starts during proestrus, and the consequent shift in follicular steroid production towards P4 synthesis (Kowalewski 2018, Kowalewski, et al. 2015). Indeed, the degree of preovulatory luteinization appears to be stronger in the dog than in other domestic mammals (Bischoff 1845, Concannon 2009, 2011, Feldman and Nelson 2004, Kowalewski 2017, Kowalewski, et al. 2015). The LH surge occurs at the end of proestrus. Spontaneous ovulation is observed approximately 24-72h after the LH surge, triggered by the decrease in the E2:P4 ratio, and takes place at circulating P4 levels of at least 5 ng/ml (Concannon, et al. 1989, Feldman and Nelson 2004, Kowalewski 2018). This marks the ovarian beginning of diestrus (also called metestrus in some publications), characterized by the presence of luteal activity and P4 dominance (Concannon, et al. 1989, Feldman and Nelson 2004, Hoffmann, et al. 1992, Kowalewski 2018). However, clinical signs of the transition from estrus to diestrus are observed later, with the termination of estrus behavior and a change in vaginal epithelial cytology appearance from superficial cells to intermediate or parabasal cells (Concannon 2011, Feldman and Nelson 2004, Kowalewski 2017). Taking into account the delay between endocrinological and clinical signs, for research purposes, the time of ovulation is considered the beginning of diestrus (Kowalewski 2017).

In the dog, the CL exhibits prolonged activity through the whole of diestrus, being the sole source of P4 in both pregnant and non-pregnant cycles as the placenta does not produce steroids (Hoffmann, et al. 1994, Nishiyama, et al. 1999). With the progression of diestrus, the highest levels of P4 are reached between days 15 to 30 post ovulation (p.o.), averaging 30 to 35 ng/ml, but with some animals exhibiting levels as high as 90 ng/ml (Concannon 2011, Concannon, et al. 1989, Hoffmann, et al. 1992). Afterwards, P4 levels start to decrease, accompanying the passive slow regression of the CL (Concannon, et al. 1989, Feldman and Nelson 2004, Kowalewski 2014). In pregnant animals, late activity of the CL (while undergoing regression) is suddenly interrupted around day 60 of its lifespan, inducing a steep decrease in circulating P4 to basal levels

(Concannon, et al. 1989, Feldman and Nelson 2004, Kowalewski 2014). The prepartum luteolytic cascade is induced by increased PGF2α produced mainly by the fetal placenta, terminating diestrus and allowing parturition to occur (Gram, et al. 2013, Gram, et al. 2014, Hoffmann, et al. 1994, Kowalewski, et al. 2010, Luz, et al. 2006).

In contrast to what is observed in livestock, the absence of pregnancy in the dog is not decisive for luteal maintenance and function. Interestingly, the constitutive expression of FP/PTGFR in the canine CL makes it sensitive to exogenously applied PGF2α, despite the high dosage and repetitive treatment required for termination of luteal function (Kowalewski, et al. 2008b, Romagnoli, et al. 1991). However, the intra-CL levels of PGF2α are low and the existence of a uterine luteolysin (PGF2α) in the absence of pregnancy has been ruled out, as hysterectomy does not affect ovarian function (Hoffmann, et al. 1992, Kowalewski, et al. 2009a, Kowalewski, et al. 2008b).

During most of diestrus, P4 circulating levels in pseudopregnant bitches are similar to those in pregnant animals (Steinetz, et al. 1989). As in the presence of pregnancy, concentrations of P4 in non-pregnant dogs start to decrease slowly after day 35 p.o., associated with regression of the CL (Concannon, et al. 1989, Feldman and Nelson 2004, Kowalewski 2014). The passive luteal regression/degeneration is associated with P4 levels fading out slowly until basal levels below 1ng/ml in peripheral plasma are reached, signaling the cycle entering the stage of anestrus (Concannon 2011, Concannon, et al. 1989, Kowalewski 2014).

Thus, by lacking an active luteolytic mechanism, non-pregnant bitches exhibit a physiological pseudopregnancy with a long-lasting luteal activity, frequently longer than in pregnant animals (Feldman and Nelson 2004, Hoffmann, et al. 1992, Kowalewski, et al. 2013).

Regarding E2 in diestrus, following the postovulatory drop, circulating levels increase again around day 10 after ovulation and appear to follow the secretion patterns of P4 [(Fig. 2) (Concannon 2011, Concannon, et al. 1989)]. However, they never reach their preovulatory concentrations (highest concentrations 45-120 pg/ml), remaining between 15-40 pg/ml (Concannon 2009, Feldman and Nelson 2004, Kowalewski 2018). Also, contrasting with other domestic animals, no pregnancy and/or parturition-associated increase of E2 is observed, the latter pointing towards the luteal source of estrogens (Concannon 2011, Kowalewski 2017, 2018).

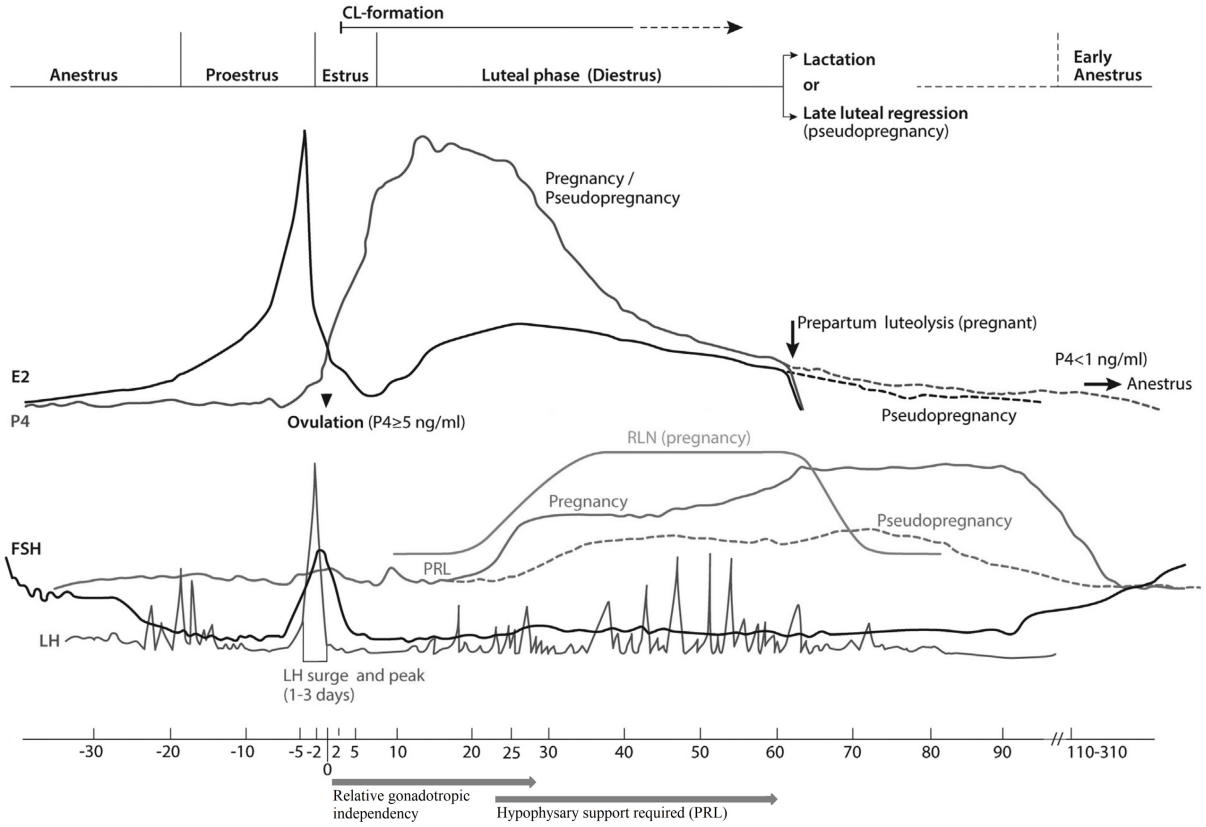

Figure 2. **Representation of the main endocrinological features of the canine reproductive cycle** (adapted from Kowalewski 2018). Day 0 marks the day of ovulation. *"Relative gonadotropic independence"* and *"Hypophysial support required"* relate to the need for gonadotropins for the maintenance and function of the CL throughout diestrus.

1.3.2. Luteal development

As the only source of P4, expectedly, the canine CL depends on several factors and mechanisms to maintain its normal function. As in other species, it relies on a dense vasculature for its development and function. During the canine early luteal phase, the expression of VEGF-system members is increased in vascular and steroidogenic cells in both pregnant and non-pregnant animals (Gram, et al. 2015a, Mariani, et al. 2006). This expression pattern appears to reflect the high angiogenic activity required for luteal development, while suggesting the presence of a cross-talk between steroidogenic and vascular cell populations. In parallel with the increased vascular and metabolic activity of the CL, the upregulation of glucose transporter 1 (GLUT1/SLC2A1) occurs, further indicating the need for an increased blood flow to fulfil CL metabolic needs (Kowalewski 2017, Papa, et al. 2014). This can also be seen in the expression patterns of other vasoactive factors, such as ETs (Gram, et al. 2015b). In fact, the increased expression of the endothelin converting enzyme 1 (ECE1), a regulator of ET availability, is accompanied by upregulation of ET2 and of the vasodilator endothelin receptor B (ETB) in the early luteal phase (Gram, et al. 2015b). Also, members of the ANGPT-system, which are regulators of vascular stability, were detected in the CL of pregnant bitches, with the expression of ANGPT1 and Tie1 decreasing during prepartum luteolysis (Gram, et al. 2018). Interestingly, besides their localization in luteal and/vascular cells,

both ANGPTs and Tie1 were also identified in macrophages, suggesting the involvement of the immune system in angiogenic and vascular processes in the dog (Gram, et al. 2018). In these regards, CL development is characterized by an increased invasion by CD4, CD8 and MHCII positive cells, mainly macrophages and lymphocytes, accompanied by an increased expression of immune factors like endoglin (END), tumor necrosis factor α (TNFα) and its receptors (Hoffmann, et al. 2004a, Nowaczyk, et al. 2017). After decreasing during mid-diestrus, a new infiltration of MHCII and CD8 positive cells can be observed in the regressing CL, while an increased expression of MHCII, TNF receptor 2 (TNFR2), IL1β, CCL3 and CCL13 is found during prepartum luteolysis (Hoffmann, et al. 2004a, Nowaczyk, et al. 2017, Zatta, et al. 2017). In addition, the expression of different interleukins, such as IL8, IL10 and IL12, and other cytokines, like transforming growth factor β (TGFβ), were previously identified by qualitative PCR in the canine CL (Engel, et al. 2005). Nevertheless, the roles of these immunoactive cells and factors in the canine CL are still not fully elucidated.

1.3.3. Luteotropic support – Gonadotropins and Progesterone

Hypophysial support is important for the function of the canine CL, as both LH and PRL exhibit luteotropic effects in the dog (Concannon 1980, Okkens, et al. 1990, Okkens, et al. 1986, Onclin, et al. 2000). In particular, PRL is an indispensable factor for the maintenance of CL function after approximately day 25, as the disruption of PRL signaling with bromocriptine (an agonist of dopamine receptors) decreases luteal production of P4 and induces premature CL degeneration (Okkens, et al. 1990, Onclin and Verstegen 1997, Onclin, et al. 2000). Also, these effects can be reversed with exogenous PRL administration, but not with LH (Okkens, et al. 1990). In the early stages, however, hypophysial support appears not to be required in the dog, as hypophysectomy had low to no effects on CL function in the first 2 – 4 weeks after ovulation (Okkens, et al. 1986). Consequently, the canine CL appears to be at least in part independent of gonadotropins in its early stages (Fig. 2).

Interestingly, the increasing availability of PRL through diestrus does not prevent CL regression, suggesting that this hypophysial hormone has a supportive role rather than being an active stimulator of CL function (Concannon, et al. 2009, Okkens, et al. 1990, Onclin and Verstegen 1997, Onclin, et al. 2000). However, the programmed regression of the CL, in combination with its independence from gonadotropins during development, suggests that the CL lifespan is inherently regulated through some local auto/paracrine mechanisms. In these regards, the expression of the nuclear P4 receptor (PGR) and estrogen receptors (ERα/ESR1 and ERβ/ESR2) in the canine CL throughout diestrus suggests a possible regulatory role of these hormones (Hoffmann, et al. 2004b). In fact, P4 appears to have luteotropic effects in the dog as the disruption of PGR signaling with

antigestagens (i.e., mifepristone or aglepristone) induces preterm luteolysis and/or abortion (Baan, et al. 2005, Hoffmann, et al. 2004b, Kowalewski, et al. 2009a, Kowalewski, et al. 2010). Auto/paracrine effects of estrogens in the canine CL were also previously proposed, but, despite some efforts being made, their role in luteal function remains elusive (Hoffmann, et al. 2004b, Papa and Hoffmann 2011).

1.3.4. Luteotropic role of prostaglandins in the dog including study with Previcox

The expression of COX2 and PTGES is observed in the luteal tissue through diestrus (Kowalewski, et al. 2009a, Kowalewski, et al. 2008a, Kowalewski, et al. 2006). Their expression is higher in the early gonadotropin-independent CL than during luteal regression (Kowalewski, et al. 2009a, Kowalewski, et al. 2008a, Kowalewski, et al. 2006). In addition, the canine CL is also sensitive to PGE2 throughout its life span, expressing both PTGER2 and PTGER4 (Kowalewski, et al. 2009a, Kowalewski, et al. 2008a). This prompted the idea that PGs, in particular PGE2, could perform luteotropic actions in this species. Indeed, such actions were observed in early canine luteal cells stimulated *in vitro* with PGE2, which exhibited increased expression of STAR and higher P4 output (Kowalewski, et al. 2013).

The decisive clues regarding the luteotropic effects of PGs in the dog came from our *in vivo* study, in which withdrawal of luteal PGs was achieved by the administration of firocoxib (Previcox®, Merial), a COX2-specific inhibitor, to bitches during the first half of diestrus (Janowski, et al. 2014, Kowalewski, et al. 2015). With this treatment, the expression of PTGES and the intra-CL levels of PGE2 were decreased during the period of CL independence from gonadotropins, demonstrating the connection between COX2 and the PTGES-dependent synthesis of PGE2 in the canine CL (Kowalewski, et al. 2015). This also showed the effectiveness of treatment in decreasing intra-CL PGs. In addition, treated animals displayed lower circulating levels of P4, accompanied by decreased expression of STAR and 3βHSD (Janowski, et al. 2014, Kowalewski, et al. 2015). Noteably, the effects on P4 levels were rather variable and, despite the decreased activity, CL function was not terminated by COX2-inhibition (Janowski, et al. 2014). Besides these effects on steroidogenic properties, other, indirect, effects of PGE2 in the canine CL were described. Accordingly, *in vitro*, PGE2 modulated the expression of different vascular factors in canine luteal cells, like ET2, ETB, ANGPT1 and ANGPT2 (Gram, et al. 2015b, Gram, et al. 2018). Also, modulatory effects of PGs regarding hormonal sensitivity of the CL were observed. *In vivo*, PRLR was downregulated following PGs withdrawal and PGE2 could upregulate this receptor in canine luteal cells *in vitro* (Kowalewski, et al. 2015). Finally, nuclei from luteal cells were smaller after

treatment with Previcox, suggesting that PGs withdrawal also affected the transcriptional activity of luteal cells (Janowski, et al. 2014).

Cumulatively, the observations so far indicate the presence of direct (on steroidogenesis) and indirect (on vascularization, hormonal sensitivity and transcriptional activity) effects of PGE2, and possibly other PGs, in the regulation of canine luteal physiology.

Cumulatively, the dog exhibits a unique reproductive cycle when compared with other domestic mammals, with the CL providing a central role in the regulation of canine reproduction. Thus, a good understanding of CL physiology is fundamental for the study of canine reproduction.

As discussed above, the regulation of CL activity can be divided into two main parts: an early developing CL independent of gonadotropic support, and a mature/regressing CL that requires primarily the support of PRL for maintenance of its function. During the early stage, PGs, mainly PGE2, play an important role in the regulation of CL function. This independent phase also appears to present the dog as an interesting model to study CL regulation without the presence of gonadotropins as major regulatory factors. Nevertheless, a better understanding of the luteotropic roles of PGs in CL function is still required

2. Hypotheses

Based on the previously observed effects of *in vivo* suppression of PGs on canine CL function (Janowski, et al. 2014, Kowalewski, et al. 2015), as well as the *in vitro* effects of PGE2 in steroidogenesis (Kowalewski, et al. 2013), the general hypothesis pursued in this PhD work is that:

(1) Prostaglandins, mainly PGE2, exert biological effects on the *corpus luteum* of the domestic dog, indicating an important luteotropic role during early diestrus.

Adding to the direct effects of PGE2 in the steroidogenic activity of the canine CL, indirect effects could also be observed including impairment of the CL sensitivity to PRL (Kowalewski, et al. 2015) and modulatory effects on the expression of vascular factors (Gram, et al. 2015b, Gram, et al. 2018). Finally, PGE2 has been described in both pro- and anti-inflammatory mechanisms in several tissues (Kalinski 2012, Nakanishi and Rosenberg 2013). For these reasons, we further hypothesize that:

(2) PGE2 might perform broader modulatory roles in the early canine CL besides the cAMP-dependent regulation of steroidogenesis, in particular regarding CL vascularization and immune system

Finally, the withdrawal of PGs by application of Previcox affected CL function but did not induce termination of CL activity (Janowski, et al. 2014, Kowalewski, et al. 2015). Thus, we also hypothesized that:

(3) Mechanisms compensating for the withdrawal of PGs might be present in the canine CL.

3. Aims

Luteal samples from the previous *in vivo* study (Janowski, et al. 2014, Kowalewski, et al. 2015) were available and used in the current PhD work. In this study, bitches from different breeds and ages were monitored for the onset of spontaneous ovulation and then allotted to either control or treated groups. From the day of ovulation, bitches were treated daily with 10 mg/kg of firocoxib (Previcox®, Merial) administered orally (treated groups) or receiving a placebo (control groups). Treatments were applied for 5, 10, 20 or 30 days p.o. On the last day of treatment, the ovaries were collected by ovariohysterectomy (OHE) and the *corpora lutea* were dissected and processed for histological or molecular analyses (n=3-5 animals/group). In addition, samples from the day of ovulation (day 0) were also available (n=5). Thus, using these samples, the following aims were defined to validate the described hypotheses:

Aim 1: Investigation of the spatio-temporal expression and localization of selected vasoactive, immunoactive and steroidogenic factors in the early canine CL during the normal cycle and in Previcox-treated animals.

This aim was accomplished by evaluating differences in the expression of several target genes in response to treatment in samples from days 0, 5, 10, 20 and 30 p.o. Selected genes included vascular factors (members of the VEGF-, ANGPT- and ET-systems) and immunoactive factors (CD4, CD8, MHCII, IL1β, IL6, IL8, IL10, IL12a, END, TGFβ and members of the TNFα family). Additionally, possible modulatory functions of PGs on the following functional groups of genes were assessed: estrogen receptors (ERα/ESR1 and ERβ/ESR2), regulators of STAR expression (cJun, SF1, YY1, GATA4 and GATA6) and of the cell cycle (CCNA2). The effects on gene expression were evaluated by applying semi-quantitative real time (TaqMan) qPCR (RT-qPCR), and were further validated at the protein level by immunohistochemistry (IHC). This allowed localization of these targets within the CL of treated and control animals. In addition, by having available control samples from different stages of luteal development, i.e., early gonadotropin-independent CL (days 5 and 10), transition to dependence on hypophysial support (day 20) and the mature CL dependent on PRL (day 30), time-dependent changes in the expression of all factors during the first half of diestrus were investigated.

Aim 2: Characterization of transcriptional changes observed in the canine CL during early diestrus and in response to the withdrawal of PGs

In order to achieve this aim, deep sequencing analysis of mRNA (RNA-Seq) from canine *corpora lutea* obtained from the Previcox study was performed with Next Generation Sequencing (NGS; deep RNA sequencing, RNA-Seq). The effects of treatment were evaluated by comparing samples from control and treated dogs at each analyzed time-point. In addition, time-dependent effects in luteal transcriptional patterns were evaluated by comparing control samples from early developing CL (days 5 and 10) with samples from the mature and fully developed CL (days 20 and 30). Identified differentially-expressed genes (DEGs) were grouped by their biological functions. Specific signaling pathways and functional networks were analyzed allowing prediction of those pathways that were more affected by treatment, together with their specific upstream regulators. Broad and multidirectional effects of treatment were observed, being also stage-dependent. Further, genes affected by treatment that might be involved in the response to the withdrawal of PGs were identified, providing a basis for future, more detailed and targeted studies.

4. Results

4.1. Manuscript 1: Prostaglandin-mediated effects in early canine *corpus luteum*: *In vivo* effects on vascular and immune factors

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Contribution:

Miguel Tavares Pereira was involved in developing the concept of the present study, experimental design, generating data, analysis and interpretation of data, and drafting of the manuscript.

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Prostaglandin-mediated effects in early canine corpus luteum: *In vivo* effects on vascular and immune factors

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ABSTRACT

Prostaglandins (PGs) are important regulators of the early corpus luteum (CL) in the dog. Whereas, initially, CL is gonadotropin independent, in the second half of its lifespan, hypophyseal support is required. The transition period is marked by decreased availability of PGs, in particular of PGE2. We previously reported that inhibition of COX2/PTGS2 *in vivo* suppressed luteal production of PGE2, lowered circulating progesterone and negatively affected luteal development. Therefore, bitches were treated with a COX2-specific blocker, firocoxib, for 5, 10, 20 and 30 days after ovulation, leading to suppression of the steroidogenic machinery. Control groups received a placebo for the same periods. Considering the wide range of possible modulatory roles of PGs shown in different organ systems, this follow-up project aimed to understand further possible PG-mediated effects in early canine CL. Thirty-four (34) factors related predominantly to vascularization and immune response were screened (mRNAs and proteins) on samples from the above described *in vivo* study. Most of the effects were observed during the transitional period (days 20 and 30). The inhibition of COX2 diminished the expression of angiopoietin family members ANGPT1, -2, Tie1 and -2 receptors. The expression of endothelin (ET)-1 was increased. Concerning the immune system, increased expression of the pro-inflammatory cytokines, IL1β, IL6 and IL12a, and elevated expression levels of CD4, was observed. Cumulatively, besides its involvement in regulating steroidogenesis, our results indicate a broader role of PGs in the canine CL, including modulation of angiogenesis, vascular stabilization and local immunomodulation. Possible cross-species translational effects are strongly implied.

1. Introduction

The corpus luteum (CL) is a temporary endocrine gland which, by producing progesterone (P4), exerts important roles in the reproduction of mammals. It is formed from the remnants of the ruptured follicle and grows rapidly to ensure an adequate steroid supply for the establishment and maintenance of pregnancy. This rapid development and steroidogenic activity require the support of rapidly developed and dense vascularization [1,2]. Also, immune system-derived factors appear to have an important role in CL formation and function [3]. Prostaglandins (PGs) are among the most prominent regulators of CL function across mammalian species, with PGE2 and PGF2α typically playing opposing roles. Thus, the luteotropic function of luteal PGE2 is mostly opposed to the luteolytic effects of PGF2α. The function of PGE2 is mostly concerned with the cAMP/PKA-mediated stimulation of STAR (steroidogenic acute regulatory protein) expression and function [4,5]. Interestingly, however, some other effects of PGE2 in the CL have been described lately, e.g., with regard to vascularization. Accordingly, e.g., in the pig, PGE2 increases the secretion of luteal VEGF [6].

In the domestic dog, circulating progesterone (P4) originates in the CL, both in pregnant and non-pregnant bitches, thereby acquiring a central role in the regulation of diestrus/pregnancy [7]. When compared with other domestic animal species, canine luteal physiology appears quite peculiar. Thus, in contrast to livestock, a uterine luteolysin (PGF2α) does not exist in non-pregnant bitches, as clearly shown in hysterectomized females which maintain their normal ovarian

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function [8]. Similarly, the presence of an intraluteal luteolytic principle can be ruled out, as there is no luteolysis in the absence of pregnancy in the dog. The intraluteal levels of PGF2α are low [9,10], but the respective receptor (FP/PTGFR) appears, however, to be constitutively expressed [9,11], reaching its highest abundance in the second half of diestrus, and rendering the canine CL receptive to exogenously applied PGF2α. Consequently, the lack of luteolysis in non-pregnant, cyclic bitches results in a prolonged pseudopregnancy, frequently lasting longer than pregnancy itself [4,8]. With this, cumulatively, the luteal regression in non-pregnant dogs appears to be a long-lasting process of structural remodelling and functional degeneration, devoid of an active luteolytic principle [5]. Conversely, in pregnant dogs, the cessation of luteal function is associated with an increased presence of utero-placental PGF2α in the maternal circulation, strongly implying its luteolytic function [7,12].

With regard to species-specific regulatory mechanisms, maintenance of the mature canine CL depends strongly on hypophyseal hormones, with prolactin (PRL) acting as the predominant luteotropic factor during the second half of diestrus, starting around 4 weeks after ovulation [13–15]. During earlier stages of the luteal phase, hypophyseal support does not seem necessary [14] and, at least in part, the CL is gonadotropin independent. At this time, increased expression of luteal cyclooxygenase 2 (COX2/PTGS2), as well as of PGE2 synthase (PTGES) and two cAMP/PKA-mediating PGE2 receptors (EP2/PTGER2 and EP4/PTGER4) in luteal cells, suggests the importance of PGs and in particular of PGE2 as autocrine/paracrine regulators of early CL function in the dog [16,17]. Supporting this idea, *in vitro* stimulation of early canine luteal cells with PGE2 increases the expression of STAR protein and potentiates P4 synthesis [4]. In our previously published studies, the luteotropic role of PGs in the early canine CL was further substantiated *in vivo* [10,18]. Thus, bitches treated with a specific inhibitor of COX2/PTGS2, firocoxib, for up to 30 days after ovulation, exhibited lower levels of intra-luteal PGE2, accompanied by downregulated expression of PTGES and steroidogenesis-linked factors (STAR and 3βHSD), and lowered circulating levels of P4 [10,18]. Interestingly, in a parallel *in vitro* experiment, it was shown that PGE2 positively regulates expression of the prolactin receptor (PRLR) in canine lutein cells. With that previous study [10], a causality has been indicated between PTGS2/COX2 function and the PTGES-dependent synthesis of PGE2, and luteal P4 synthesis in the dog.

As mentioned elsewhere, the development and activity of CL rely on rapid development of a dense vascular network. Accordingly, also in the dog, the luteal expression of VEGFA and VEGFR1 is upregulated in early CL of pregnant and non-pregnant bitches [19,20]. Furthermore, vasoactive factors from the endothelins family are present in the canine CL and are expressed in a time-dependent manner throughout the CL life span [21]. Thus, the early luteal phase is characterized by elevated levels of endothelin 2 (ET2), endothelin receptor B (ETB) and the endothelin converting enzyme 1 (ECE1) that regulates the availability of endothelins [21]. It is noteworthy in this context that, in one of our previous studies, PGE2 was shown to upregulate *in vitro* luteal expression of the vasodilatory endothelin receptor B (ETB) in the dog [21]. The effects of PGE2 on the expression of VEGFA and the endothelin-system have, however, never been assessed *in vivo*. The functionality of the vasculature appears to be also affected during prepartum luteolysis, as implied by the increased expression of vasoconstrictive endothelin A receptor (ETA) [21]. No such effects could be observed for the CL of non-pregnant dogs during late luteal regression [21,22].

With regard to the immune system, apart from its indicated involvement in termination of luteal function in pregnant dogs [22], the development of the CL is also associated with increased activity of immune system-derived factors. This is indicated by the increased infiltration of immune cells [23], as well as increased expression of different chemokines (e.g., tumor necrosis factor alpha; TNFα [24]). Accordingly, increased numbers of CD4, CD8 and endoglin (END) positive cells were observed in the CL of pregnant and non-pregnant cycles [23,24] during luteal formation. Additionally, the expression of several cytokines has been confirmed in canine luteal tissue, e.g., interleukin (IL) 8, IL10, IL12a, TNFα or transforming growth factor 1 beta (TGF1β) [24–26]. Nevertheless, the exact role of these factors in the development and maintenance of the canine CL remains to be elucidated.

In this context, adding to the possible roles of PGE2 in maintaining tissue homeostasis, both pro- and anti-inflammatory mechanisms have been described for this prostaglandin in different tissues [27,28]. In the reproductive system, PGE2 was shown to modulate immune activity in a suppressive manner in the human uterus [29]. These effects seem to support feto-maternal immune tolerance. In other tissues, e.g., the lung, PGE2-induced immunomodulation is linked to control of inflammation and limitation of damage during prolonged immune responses [27,30]. However, to the best of our knowledge, nothing is known about immunomodulatory effects that PGE2 and other PGs could exert in the early canine CL.

Here, taking into account the so far known effects exerted by PGs upon different regulatory systems *in vitro*, and the effects resulting from firocoxib treatment and, thereby, the suppression of PGs function *in vivo*, we hypothesized that PGs could also have other biological effects in the early canine CL. Therefore, using the tissue material from our above mentioned previous studies [10,18], we investigated the possible impact of blocking COX2/PTGS2 on vascular and immune functions during establishment and development of the canine CL. The expression of 34 different potential target genes known for their involvement in regulating CL function was screened.

2. Materials and methods

2.1. Tissue samples

This is a follow up project utilizing tissue samples obtained in our previously described *in vivo* study [10,18], approved by the responsible ethics committee of the University of Warmia and Mazury in Olsztyn, Poland (permit 54/2008).

In brief, middle-sized mixed breed bitches aged 2 to 7 years were observed for the onset of spontaneous estrus by vaginal cytology and P4 assay. The day of ovulation (Day 0) was considered the day when circulating P4 concentrations for the first time exceeded 5 ng/ml. The animals were then randomly assigned (simple randomization) to four treatment groups and five control groups. Bitches from treated groups received, orally, 10 mg/kg body weight per day (twice the recommended dose) of firocoxib (Previcox®, Merial Ltd), a COX2/PTGS2 specific inhibitor, for 5 (n = 4), 10 (n = 4), 20 (n = 3) and 30 (n = 5) days. Animals from control groups received a placebo for 0 (n = 5), 5 (n = 5), 10 (n = 4), 20 (n = 3) and 30 (n = 4) days. With this, samples used in our study derive from different stages of early luteal development in the dog, i.e., developing gonadotropin-independent CL (days 5 and 10), mature CL in transition between gonadotropin independency and dependency (day 20) and mature gonadotropin-dependent CL (day 30) [5,13–15]. This allowed further analysis of the effects of time on the expression of the studied target genes. On the last day of treatment, animals were ovariohysterectomized for collection of ovaries containing CLs. The corresponding P4 levels are presented in [10,18]. Ovaries were then immediately trimmed of surrounding connective tissue and divided into two parts. CLs from one part were preserved for mRNA analysis, being placed in RNAlater (Ambion Biotechnology GmbH, Wiesbaden, Germany) for 24 h at 4 °C and then stored in −80 °C until further use. The second half of the ovary was fixed in 10% phosphate-buffered formalin for 24 h at 4 °C and then paraffin embedded using standard methodology.

2.2. Total RNA extraction, high capacity reverse transcription (RT) and pre-amplification of cDNA

Extraction of total RNA from all samples was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA concentration and purity were measured with a Nano Drop 2000C spectrophotometer (Thermo Fisher Scientific AG, Reinach, Switzerland).

Samples were diluted and a total amount of 10 ng of RNA was used from each sample. To eliminate possible genomic DNA contamination, all samples were treated with the RQ1 RNase-free DNase kit (Promega, Dübendorf, Switzerland) following the manufacturer's protocol. Reverse transcription (RT) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Foster City, CA, USA), following the supplier's protocol, obtaining cDNA in a final volume of 20 µl. Finally, the amount of cDNA was amplified with the TaqMan® PreAmp Master Mix Kit (Applied Biosystems). For this, pooling of TaqMan assays for all selected target genes and reference genes was prepared in a 250 µl mix containing: 180 nM of each forward and reverse primer and 50 nM of probe for commercially available TaqMan assays, and 15 nM of each forward and reverse primer and 20 nM of each probe for self-designed TaqMan systems. For subsequent amplification, 12.5 µl of the previously prepared cDNA from each sample was mixed with 25 µl of 2x TaqMan Preamp Master Mix and 12.5 µl of the pooled TaqMan assays mix. Enzymes were activated at 95 °C for 10 min and then samples were amplified through 14 cycles of 95 °C for 15 s and 60 °C for 4 min, each. All reactions were performed in an Eppendorf Mastercycler (Vaudax-Eppendorf AG, Basel, Switzerland).

2.3. Semi-quantitative real-time TaqMan PCR (qPCR) and data evaluation

Semi-quantification of luteal expression of 34 selected target genes was assessed by real-time (TaqMan) PCR.

For each sample and target gene, 25 µl of reaction mixture was prepared, containing 200 nM TaqMan Probe, 300 nM of each primer, 12.5 µl Fast Start Universal Probe Master (ROX®) (Roche Diagnostics, Mannheim, Germany) and 5 µl of pre-amplified cDNA obtained as described above. All reactions were run in duplicates in 96-well optical plates. Autoclaved water instead of cDNA and the minus-RT control were run as negative controls. A detailed description of primers, TaqMan probes and predesigned (i.e., commercially available) TaqMan systems, as well as all gene names and their corresponding abbreviations are presented in Suppl. File 1. Self-designed primers and 6-carboxyfluorescein (6-FAM) and 6-carbocytetramethylrhodamine (TAMRA) labelled probes were ordered from Microsynth (Balgach, Switzerland). Commercially-available TaqMan systems were obtained from Applied Biosystems. Efficiency values of the PCR reactions were validated to ensure approximately 100%. For this, the CT slope method was performed, using as the template cDNA obtained from CL of two different stages and each diluted in a 10-fold series, according to the instructions of the manufacturer and as previously described [31]. Real-time PCR reactions were run in an automated ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Reactions were activated at 95 °C for 10 min, and then samples were amplified in 40 cycles comprising of: 95 °C for 15 s and 60 °C for 60 s, each. Results were quantified with the comparative CT (ΔΔCT) method according to the protocol provided by the manufacturer of ABI PRISM 7500 and as described previously [16,31]. The expression of the reference genes was used for normalization of target gene expression and the lowest expression for each target gene was used as a calibrator. As for reference genes, in pilot experiments, three genes (*GAPDH*, *β-ACTIN* and *CYCLOPHILIN A*) were initially evaluated, based on their expression in all samples included in the study. Then, evaluation of reference gene stability was performed using two programs: geNorm and NormFinder [32,33]. Finally, *GAPDH* was selected as the most stable gene, compared with *β-ACTIN* and *CYCLOPHILIN A*, and was used as the reference gene for the ΔΔCT evaluation.

To evaluate the effects of treatment on the expression of target genes, an unpaired, two-tailed Student's *t*-test was used, comparing treatment group with control group, from each time point (day). Additionally, time-related changes in the expression of all target genes in control animals were evaluated using Kruskal-Wallis (non-parametric ANOVA) followed by Dunn's test. All numerical results for relative gene expression were logarithmically transformed and are presented as geometric means (Xg) +/- geometric standard deviation (SD). All statistical tests were performed with GraphPad3 (GraphPad Software Inc., San Diego, CA, USA); $P < 0.05$ was considered statistically significant.

2.4. Immunohistochemical staining

Depending on the availability of canine species-specific and/or cross-reacting antibodies, immunohistochemistry (IHC) was performed for those factors that showed a significant response to treatment at the mRNA level. Thus, staining was performed against members of the ANGPT system (ANGPT1, ANGPT2, TIE1 and TIE2), ET1 and CD4; more details are listed below. Antibodies targeted against IL1β (NB600-633, Novus Biologicals LLC, Littleton, CO, USA) and IL12a (Orb256618, Biorbyt Ltd., Cambridge, UK) were also tested, but failed to produce reliable staining. No commercially-available antibody against IL6 with described/predicted cross-reactivity for the canine species was found.

The standard, indirect immunoperoxidase staining method was used for IHC, following our previously published protocol with canine tissues [16]. Briefly, formalin-fixed and paraffin-embedded luteal tissues were cut on a microtome (2–3 µm thick sections), transferred to SuperFrost microscope slides (Menzel-Glaeser, Braunschweig, Germany) and dried overnight at 37 °C. At least 5–6 slides were analysed from each animal. The tissue slices were then deparaffinized with xylene and rehydrated in a graded ethanol series, ending with tap water. Antigen retrieval was obtained by microwave heating in citrate buffer (pH = 6) for three cycles of 5 min at 600 W. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Slides were washed in IHC buffer/0.3% Triton X pH 7.2–7.4 (0.8 mM Na_2HPO_4, 1.74 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl), and incubated in 10% normal horse serum (for TIE2, ET1 and CD4), or 10% normal goat serum (for ANGPT1, ANGPT2 and TIE1), for 20 min at ambient temperature to reduce non-specific binding. Additional blocking with 1.5% bovine serum albumin was performed for 5 min for CD4. Finally, samples were incubated overnight at 4 °C with the following primary antibodies at respective dilutions: anti-ANGPT1 (PAA008Ca01, Cloud-clone Corp., Houston, TX, USA), 1:50; anti-ANGPT2 (TA343276, OriGene Technologies Inc., Rockville, MD, USA), 1:400; anti-TIE1 (sc-9025, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 1:100; anti-TIE2 (sc-31268, Santa Cruz Biotechnology Inc.) 1:200; anti-ET1 (SIGMA-E166, Sigma-Aldrich, St. Louis, MO, USA), 1:600; anti-CD4 (GTX84720, GeneTex, Irvine, CA, USA), 1:600. Afterwards, samples were incubated in secondary antibody diluted 1:100 (BA-100 biotinylated goat anti-rabbit IgG for ANGPT1, ANGPT2 and TIE1; BA-9500 biotinylated horse anti-goat IgG for TIE2; BA-2000 biotinylated horse anti-mouse IgG for ET1 and CD4; all from Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at ambient temperature, followed by incubation with streptavidin-peroxidase Vectastain ABC kit (Vector Laboratories Inc.) for 30 min at room temperature. Signals were developed with the Liquid DAB + substrate kit (Dako Schweiz AG, Baar, Switzerland). For each antibody, all groups were simultaneously stained using the same development time. Slides were counterstained with hematoxylin, dehydrated in a graded ethanol series and mounted in Histokit (Assistant, Osterode, Germany). For negative controls, sections were incubated with non-immune IgG instead of primary antibodies, from the same species and at the same concentration as the primary antibody (isotype control): rabbit IgG (I-1000) for ANGPT1, ANGPT2 and TIE1, goat IgG (I-5000) for TIE2 and mouse IgG (I-2000) for ET1, all from Vector

Laboratories Inc., and mouse IgG1 for CD4 (ABIN376357, Antibodies-Online GmbH, Aachen, Germany). An additional control was comprised of sections omitting the primary antibody.

Slides were evaluated under a Leica DMRXE light microscope equipped with a Leica DFC425 camera (Leica Microsystems, Wetzlar, Germany). IHC slides were assessed qualitatively by two independent researchers for localization of the respective target proteins and representative pictures were taken. Morphological identification of cellular components within the CL, including immune cell differentiation, was based on available histologic literature [34,35], as well as on information available from the canine literature, and on the herein observed luteal distribution of CD4 expressing cells that corroborates previously published findings [5,23,24,36]. In particular, the localization of CD4 positive signals could be predicted by their typical cellular localization, which was described previously for the dog as being localized in monocytes and macrophages [23,24], thereby serving as a positive internal control.

3. Results

3.1. Semi-quantification of target gene expression

The expression of almost all target genes was detectable in all tissue material available. The only exception was for endothelin 3 (*ET3*), whose expression was frequently below the detection limit, preventing quantitative assessment of its expression. Also, a relatively high individual variability in the expression of some of the studied factors was observed. Despite that, as presented below, treatment and/or time-related changes in the expression of several genes were observed (Fig. 1–3 and Suppl. File 2). On the other hand, no statistically significant changes ($P > 0.05$) were observed for the following genes, neither in their responses to treatment nor during the experimental time-course: *VEGFA*, *ETA*, *MHCII*, *CD8*, *IL8*, *TGF1β*, *TNFR1* and *GATA4* (Suppl. File 3).

3.2. Effects of treatment on target gene expression

Of the 33 detectable factors, effects of Previcox® treatment on mRNA expression were observed on 11: the angiopoietin (ANGPT)-family members (*ANGPT1*, *ANGPT2*, *TIE1* and *TIE2*), as well as *ET1*, *CD4*, *IL1β*, *IL6*, *IL12a*, *cJUN* and *CCNA2*. Thus, *ANGPT1* expression was significantly decreased on day 20 in the treated group ($P < 0.05$, Fig. 1A) while *ANGPT2* ($P < 0.0001$, Fig. 1B), *TIE1* ($P < 0.04$, Fig. 1C) and *TIE2* ($P < 0.04$, Fig. 1D) were decreased on day 30 after ovulation, compared with placebo-treated controls. Conversely, *ET1* expression was increased by treatment on day 20 ($P < 0.03$, Fig. 2A). As for immune factors, whereas the expression of *CD4* was increased on day 30 ($P < 0.03$, Fig. 2B) by the treatment, mRNA levels of *IL1β* ($P < 0.04$, Fig. 2C), *IL12a* ($P < 0.002$, Fig. 2D) and of *IL6* ($P < 0.02$, Fig. 2G) were elevated on day 20. Finally, the expression of *cJUN* ($P < 0.01$, Fig. 3A) and *CCNA2* ($P < 0.01$, Fig. 3B) was also increased at day 20 after ovulation in CL of treated animals. The expression of some of the factors varied greatly in CL of treated animals, e.g., both estrogen receptors, *ERα/ESR1*, and in particular *ERβ/ESR2* or *TNFα*, but their expression levels were not significantly affected by the treatment ($P > 0.05$) (Suppl. File 2).

At the protein level, ANGPT1 was detected in endothelial cells and *tunica media* and pericytes of blood vessels (Fig. 1E). Whereas ANGPT2 was clearly detectable in luteal cells (Fig. 1F), only weak or no signals were detected for ANGPT1 in these cells (Fig. 1E). Additionally, signals for both angiopoietins were detected in interstitial cells identified as luteal macrophages. The expression of ANGPTs in the canine luteal macrophages was verified previously on consecutive sections stained for MHCII/ANGPT2 [36] in the CL of pregnancy. As for angiopoietin receptors, signals for TIE1 were observed in *tunica intima* (endothelial cells), *tunica media* and pericytes of vessels, macrophages and in luteal cells (Fig. 1G), while TIE2 was predominantly localized in vascular endothelial cells and *tunica media*/pericytes of vessels (Fig. 1H). The only member of the endothelin family affected by the treatment, ET1, was localized in luteal cells (Fig. 2E). Finally, CD4-positive cells were identified as monocytes/macrophages (Fig. 2F). Also, this localization pattern of CD4 in immune cells of canine CL was verified previously [24]. In general, the effects of treatment observed at the IHC level appeared to mirror their expression levels observed by qPCR. This was particularly obvious for ANGPT2 at day 30 and for ET1 at day 20. An apparent increase in the number of CD4-positive cells present in CL was observed, mainly in regions surrounding blood vessels.

3.3. Time-related changes in gene expression

Time-related changes in target gene expression were assessed in all control groups from the time of ovulation through early diestrus. During the observation period, the expression pattern of several factors affected by the treatment, *ANGPT2*, *TIE1*, *TIE2, CD4* and *CCNA2*, was time-dependent ($P = 0.0015$, $P = 0.0001$, $P = 0.009$, $P = 0.01$, and $P = 0.001$, respectively) (Figs. 1–3).

Over time, expression of *ANGPT2* and of its receptors, *TIE1* and *-2*, increased. The expression of *ANGPT2* and *TIE1* was significantly higher on days 20 and 30 than during early CL formation ($P < 0.05$, Fig. 1B and C), while the expression of *TIE2* was significantly higher on day 20 compared with the day of ovulation ($P < 0.05$, Fig. 1D). *CD4* expression was more stable in early luteal stages, but it was significantly higher at day 20 than day 30 after ovulation, when its expression was significantly downregulated ($P < 0.01$, Fig. 2B). Finally, representation of the cell cycle regulator *CCNA2* was relatively higher in early CL, showing the highest mRNA levels at day 5 and decreasing significantly by day 20 after ovulation ($P < 0.01$, Fig. 3B).

Time-related effects in gene expression were also observed in several target genes that were not affected by the treatment: *ETB* ($P = 0.005$), *ET2* ($P = 0.03$), *ECE1* ($P = 0.03$), *eNOS* ($P = 0.02$), *iNOS* ($P = 0.02$), *ERα/ESR1* ($P = 0.01$), *ERβ/ESR2* ($P = 0.001$), *YY1* ($P = 0.002$), *SF1* ($P = 0.004$), *GATA6* ($P = 0.002$), *END* ($P = 0.01$), *IL10* ($P = 0.03$), *TNFα* ($P = 0.008$) and *TNFR2* ($P = 0.01$) (Suppl. File 2). Additionally, summary of the respective results is presented in Table 1.

The expression of *ETB* increased rapidly after ovulation, being significantly lower at the time of ovulation than at any other evaluated time points thereafter ($P < 0.05$, Table 1, Suppl. File 2A). In contrast, *ET2* decreased after ovulation, reaching its lowest expression on day 10 ($P < 0.05$, Table 1, Suppl. File 2B). Finally, the levels of mRNA encoding for *ECE1* showed significantly increased levels on days 20 and 30 compared with the day of ovulation ($P < 0.01$) and day 10 ($P < 0.05$, Table 1, Suppl. File 2C). Functionally related to the endothelin-system, expression of *eNOS* (endothelial nitric oxide synthase) and *iNOS* (inducible NOS) was lowest on the day of ovulation, while *eNOS* expression increased significantly towards day 20 ($P < 0.05$, Table 1, Suppl. File 2D), and on days 20 and 30 for *iNOS* ($P < 0.05$, Table 1, Suppl. File 2E). The expression of *ERα/ESR1* increased significantly between the day of ovulation and day 30 ($P < 0.05$ for *ERα*), whereas *ERβ/ESR2* expression was significantly higher on day 30 than on day 10 after ovulation ($P < 0.01$) (Table 1, Suppl. File 2F and G). The levels of mRNA encoding for the transcriptional factors and known regulators of STAR expression, *YY1* and *SF1*, increased significantly after ovulation, with the highest expression on day 20 for *YY1* ($P < 0.01$, Table 1, Suppl. File 2H), and on days 20 and 30 for *SF1* ($P < 0.05$, Table 1, Suppl. File 2I). In contrast, *GATA6* exhibited significantly higher expression on day 5 than on days 20 and 30 ($P < 0.01$ and $P < 0.05$, respectively, Table 1, Suppl. File 2J). As for the immune factors, *END* mRNA levels increased significantly over time, from the day of ovulation towards day 30 ($P < 0.01$, Table 1, Suppl. File 2K). The opposite expression pattern was observed for *IL10*, *TNFα* and its receptor *TNFR2* which showed the highest mRNA abundance on day 5

after ovulation, decreasing significantly towards either day 20 (*TNFα*, $P < 0.05$, Table 1, Suppl. File 2M) or day 30 (*IL10* and *TNFR2*, $P < 0.05$, Table 1, Suppl. File 2L and N, respectively).

4. Discussion

A plethora of roles has been attributed to PGs in mammals, including those roles less studied with respect to the reproductive tract, such as immunomodulation/suppression or vasodilation and

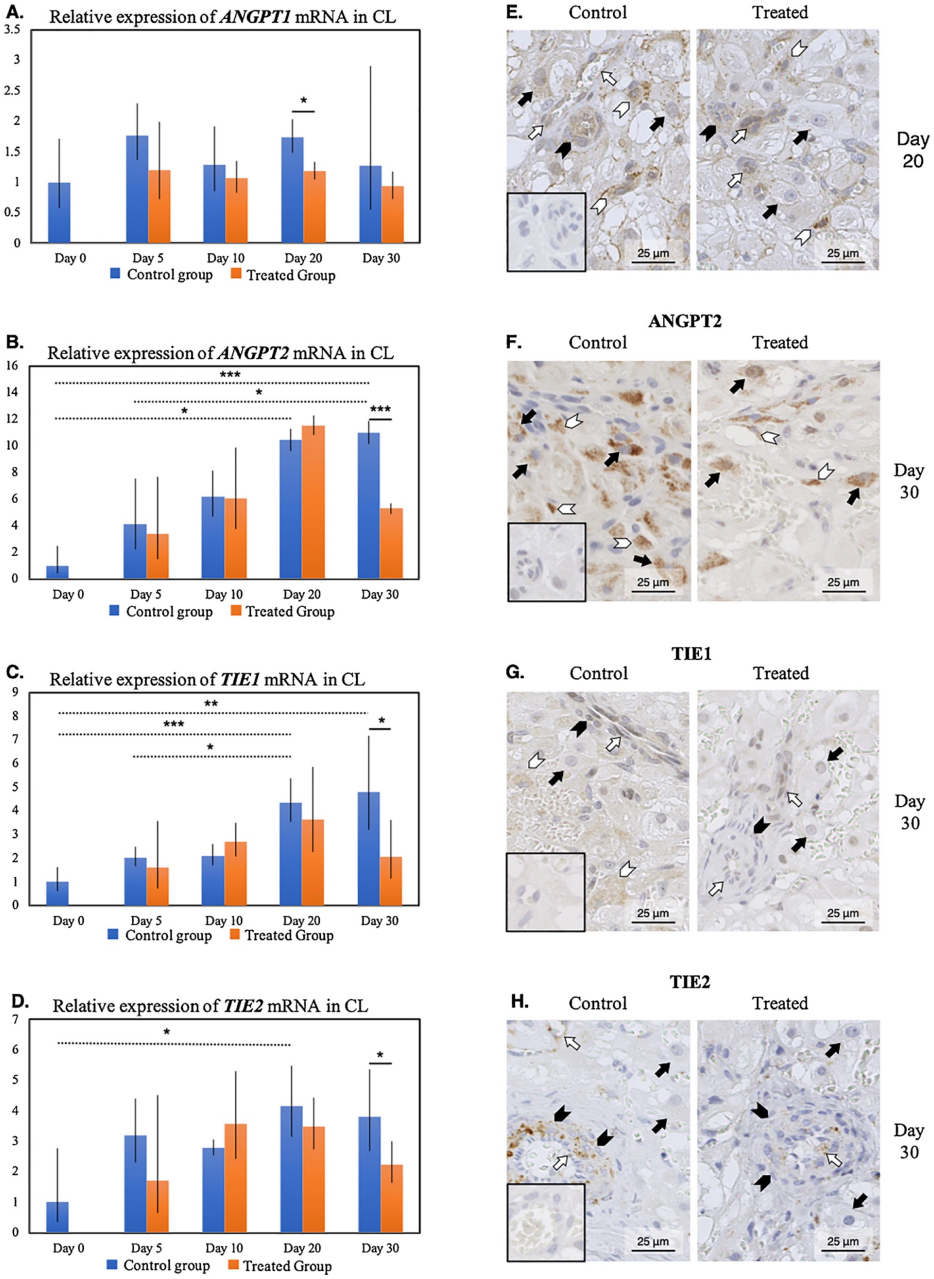

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Fig. 1. Expression of ANGPT1, ANGPT2, TIE1 and TIE2 in luteal tissue of control and Previcox-treated dogs. (A–D) Relative target gene expression as determined by Real Time (TaqMan) PCR (Xg ± SD). The Kruskal–Wallis (non-parametric ANOVA) was applied to test the effects of time on gene expression in all control samples, revealing: $P > 0.05$ for *ANGPT1,* $P = 0.0015$ for *ANGPT2*, $P = 0.0001$ for *TIE1* and $P = 0.009$ for *TIE2*. This, in case of $P < 0.05$, was followed by Dunn's multiple comparison post-test (dotted lines). Student's *t*-test was applied to test the effect of treatment on gene expression (solid lines). Bars with asterisks differ at: * $=P < 0.05$, ** $=P < 0.01$, *** $=P < 0.001$. (E–H) Immunohistochemical localization of respective proteins; representative pictures are shown at selected time points after ovulation. (E) ANGPT1 protein is detected in endothelial cells (open arrows), *tunica media* or pericytes of vessels (solid arrowheads) and macrophages (open arrowheads). Weaker or no signals are detected in luteal cells (solid arrows). (F) ANGPT2 protein is localized predominantly in luteal cells (solid arrows) and luteal macrophages (open arrowheads). (G) TIE1 localizes in endothelial cells (open arrows), *tunica media* or pericytes of vessels (solid arrowheads), macrophages (open arrowheads) and luteal cells (solid arrows). (H) TIE2 stains in endothelial cells (open arrows) and *tunica media* or pericytes (solid arrowheads). Weaker or no signals are detected in luteal cells (solid arrows). No staining is observed in the isotype control (left bottom pictures in E–H, shown at the same magnification).

vasoconstriction. As for the CL of the dog, in particular PGE2 is considered to be among the important regulators in the early, gonadotropin-independent developmental stage. Its function has been associated predominantly with cAMP-dependent regulation of steroidogenesis [5]. Consequently, based on the possible causality implied in our previous studies between COX2/PTGS2 activity and the functionality of PGE2 in Previcox-treated dogs [10,18], here, other potential biological effects exerted by PGs in the canine CL were investigated. The main focus was on the vascular and immune systems. Thus, a wide range of factors possibly involved in the regulation of luteal function was screened for their expression during the normal early luteal phase and following application of Previcox. According to our knowledge, some of these factors were thoroughly studied and/or quantitatively assessed for the first time in the early canine CL, e.g., *IL1β*, IL6, *IL12a*, *IL10, eNOS* and *iNOS*. Additionally, the expression of some other factors involved in the establishment and maintenance of CL function was examined, such as *ERα/ESR1* and *ERβ/ESR2*, or transcriptional regulators of *STAR*: *GATA4* and *-6, SF1,* c*JUN* and *YY1*. Despite large individual variations in the expression of most of the investigated factors upon treatment with Previcox (discussed in more detail later), several of them were identified as being strongly affected by PGs withdrawal.

4.1. Vascular factors

Among the vascular factors, the ANGPT system was the most strongly affected by Previcox treatment in our study. This study is the first to show expression of the ANGPT system during CL development in non-pregnant dogs. Its expression in the CL of pregnant dogs was shown recently [36]. Whereas the expression of ANGPT1 was time-dependent, showing strong decrease towards prepartum luteolysis, the expression of ANGPT2 remained highly expressed and clearly detectable over time [36]. Similarly, in the herein presented study, CL formation was associated with increasing mRNA levels of *ANGPT2* and the two receptors, *TIE1*, and *TIE2*. The expression of *ANGPT1* remained more or less stable during the 30 days of luteal development of non-pregnant dogs. Consistent with our previous findings with CL of pregnancy [36], the expression of ANGPT1 was targeted to luteal vessels, whereas ANGPT2 was localized mainly in luteal cells. Also, interstitial cells and cells identified as local macrophages [24,34–36], were identified as a possible source of ANGPTs in the canine CL. Both receptors were predominantly localized in the vascular components of the CL, pointing towards functional interplay of the ANGPT family members in regulating the canine CL. Acting together with VEGFA, and utilizing their tyrosine kinase receptors, TIE1 and TIE2, ANGPTs regulate vascular stability [37–40]. Whereas ANGPT1 promotes vascular stabilization and prevents uncontrolled angiogenesis [37,38], ANGPT2 in the presence of abundant concentrations of VEGFA (e.g., during the early luteal phase) causes destabilization/loosening of blood vessels and supports migration of endothelial cells, enhancing angiogenesis [2,41]. The results of our study indicate that the negative effects of Previcox treatment on expression of ANGPT family members point towards modulatory effects of PGs on luteal angiogenesis and vessel stabilization. Taking into consideration the cellular distribution of ANGPTs and their receptors in Previcox treated animals, we propose that PGs have stimulatory effects on the ANGPT system in different luteal cellular compartments.

Interestingly, the expression of VEGFA was not affected by the treatment in the present study. Thus, the functional connection between locally acting PGs, in particular PGE2, and the VEGF system, established previously for the pig and rat CL [6,42], does not seem to apply in the dog.

Furthermore, adding to the positive effects exerted by PGE2 on ETB expression in isolated canine lutein cells [21], here we observed modulatory effects of PGs withdrawal on ET1. In agreement with our previous study [21], ET1 in the canine CL was localized in lutein cells. Endothelins play important roles in the regulation of several ovarian functions such as vascularization, steroidogenesis, ovulation, folliculogenesis, luteal regression and luteolysis [43,44]. Besides ET1, they are represented by ET2 and ET3, and upon activation by the specific converting enzyme (ECE1), endothelins exert their ETA- and ETB-mediated effects, the latter involving activation of the nitric oxide (NO) pathway [45,46]. Here, although *ETB* expression seemed to be lowered by Previcox treatment, these changes were not statistically significant, most probably due to high individual variations.

Taking into account, however, the above-mentioned effects of PGE2 on ETB expression and the stimulatory effects exerted by PGs suppression on *ET1* expression, it seems that the role of PGs in regulating the expression and function of luteal endothelins is a topic worthy of more attention in future research. In addition, ET1 has been linked to luteolytic processes in several species, e.g., in ruminants, rats and rabbits [47–50]. Similarly, along with the vasoconstrictive ETA, the expression of ET1 was strongly increased in our previous study in which a PGR blocker was applied for pregnancy termination in mid-pregnant dogs [21].

On the other hand, in accordance with our previous findings, CL development was characterized by increased intraluteal levels of *ETB* and *ECE1* [21]. The increased vascularization observed during this time was also mirrored in increasing expression of endoglin (*END*), which is a phenomenon described previously for the CL of both pregnant and non-pregnant dogs [23,24]. Similarly, expression of the respective NO synthases, *eNOS* and *iNOS*, known for their involvement in regulating luteal function in other species, such as cattle or rats [51–53], increased significantly with progression of the luteal phase. In the present study, however, their expression was unaffected by Previcox treatment.

4.2. Immune factors

The early luteal phase in the dog is associated with increased infiltration of CD4- and CD8-positive cells, mostly representing macrophages, monocytes and lymphocytes [23,24]. Their presence and distribution within the CL of pregnant and non-pregnant dogs has been described before [23,24]. Particularly for CD4 expression, we were able to confirm these previous observations in the present study. A new finding from our study was, however, the increased expression of *CD4* in the CL of Previcox treated animals. As for other mediators of the immune response, the pro-inflammatory *IL1β* and *IL12a* did not vary strongly individually nor with regard to the stages of early CL

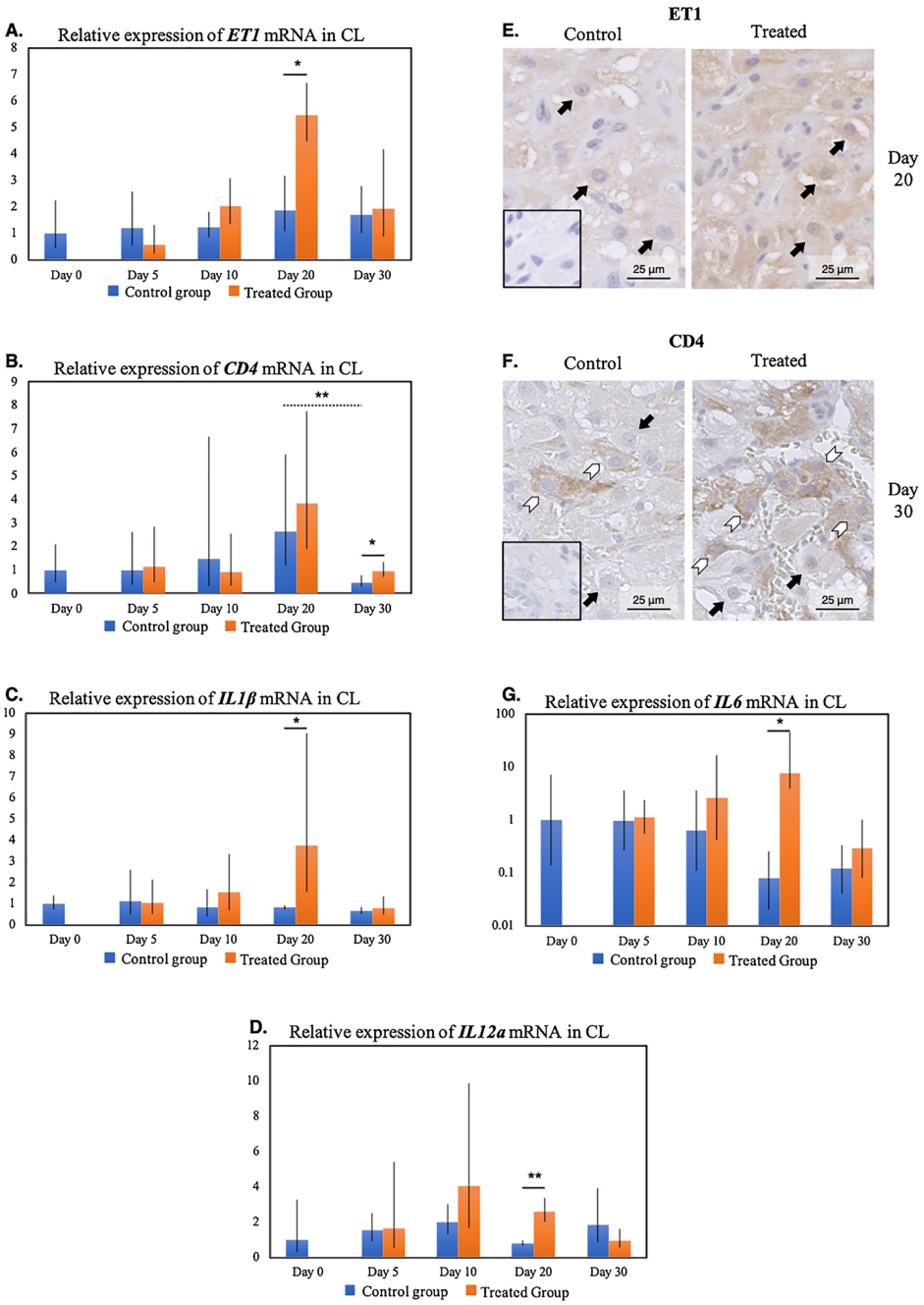

Fig. 2. Expression of ET1, CD4, IL1β, IL6 and IL12a in luteal tissue of control and Previcox-treated dogs. (A–D and G) relative target gene expression as determined by Real Time (TaqMan) PCR (Xg ± SD). The Kruskal-Wallis (non-parametric ANOVA) was applied to test the effects of time on gene expression in all control samples, revealing: $P > 0.05$ for *ET1*, *IL1β*, *IL6* and *IL12a*, and $P = 0.01$ for *CD4*. This, for CD4, was followed by Dunn's multiple comparison post-test (dotted line). Student's *t*-test was applied to test the effect of treatment on gene expression (solid lines). Bars with asterisks differ at: *=$P < 0.05$; **=$P < 0.01$. (E–F) Immunohistochemical localization of ET1 and CD4; representative pictures are shown at selected time points after ovulation. (E) ET1 protein is localized in luteal cells (solid arrows). (F) CD4 protein is localized in monocytes/macrophages (open arrowheads); lutein cells are indicated by solid arrows. No staining is observed in the isotype control (left bottom pictures in E and F, shown at the same magnification).

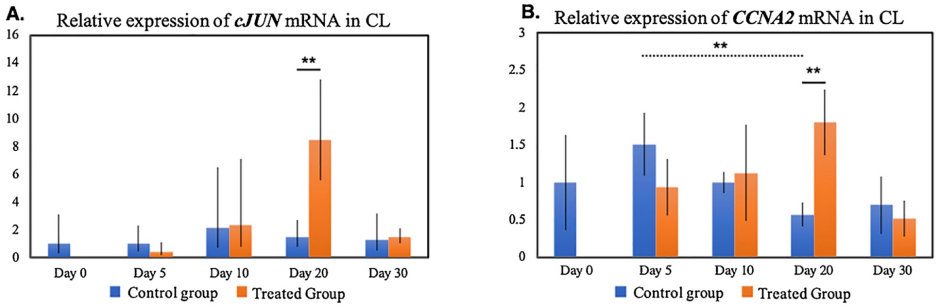

Fig. 3. Expression of *cJUN* and *CCNA2* mRNA in luteal tissue samples of control and Previcox-treated dogs. Relative target gene expression was determined by Real Time (TaqMan) PCR (Xg ± SD). The Kruskal-Wallis (non-parametric ANOVA) was applied to test the effects of time on gene expression in all control samples revealing: $P > 0.05$ for cJun and $P = 0.001$ for CCNA2. In case of CCNA2 it was followed by Dunn's multiple comparison post-test (dotted line in B). Student's *t*-test was applied to test the effect of treatment on gene expression (solid lines). Bars with asterisks differ at: ** = $P < 0.01$.

development. The expression of *IL6* seemed to be lower at day 20 and 30, but also these effects were not statistically significant. With regard to Previcox treatment, like for CD4-expressing cells, the expression of all three pro-inflammatory cytokines (*IL1β*, *IL12a* and *IL6*) was significantly increased. This was, indeed, an interesting discovery from our study as it indicates modulatory, possibly immunosuppressive, effects of PGs in the canine CL. The pro-inflammatory properties of IL1β in the CL of the dog were recently implicated in our transcriptome studies, revealing its increased presence in apoptosis-dominated CL during prepartum luteolysis [22]. Conversely, the elevated expression of *IL10* during early CL development, at day 10 after ovulation, indicates its possible luteotropic function, resembling thereby the situation described previously for human luteal cells, in which IL10 was shown to stimulate P4 production [54].

Finally, whereas the expression of *TNFR1* was not significantly affected in our study, the expression of *TNFα* and *TNFR2* was higher in early developing CL, resembling thereby their expression patterns described previously [24]. The TNF-system was, however, not modulated in response to the suppression of PGs synthesis.

4.3. Estrogen receptors

When expression of the estrogen receptors, *ERα/ESR1* and *ERβ/ESR2*, was examined, similarly to what has been observed for the *PGR* [10], their mRNA levels remained unaffected by treatment with Previcox. Time-related changes in the expression of these two receptors in the canine CL were shown previously [55]. In accordance with our findings, here, the expression of *ERα/ESR1* increased between luteal formation and mid-diestrus. However, and in contrast to the previous report, changes in *ERβ/ESR2* were also found in this study, revealing its increasing expression towards mid-diestrus. This difference could be explained by the high variability in circulating levels of 17β-estradiol (E2) [7], possibly exerting regulatory effects on its own receptors.

4.4. Factors involved in steroidogenesis

Knowing the importance of some key transcriptional factors in regulating STAR expression, we decided to assess the basic capability of luteal PGs to modulate their expression in the CL following the suppression of prostaglandin synthesis. Among the five regulators studied here (*cJUN*, *SF1*, *GATA4*, *GATA6* and *YY1*), only *cJUN* expression was affected by the treatment. The cJUN is a member of the AP1-family of transcriptional factors, and is well known for its involvement in regulating STAR promoter activity [56]. In pregnant dogs, its luteal expression was suppressed towards prepartum luteolysis, mirroring the diminishing STAR expression and circulating P4 levels at that time [22]. Knowing this, it was perplexing to observe increased expression of *cJUN* in response to Previcox treatment at day 20 after ovulation. A similar effect was observed for *CCNA2*, which encodes for cyclin-A2 protein, a positive regulator of the cell cycle [57]. Since, clearly, as observed in our previous studies, blocking COX2/PTGS2 led to a decrease in the size of luteal cell nuclei (indicating diminished transcriptional activity), lower STAR expression and lowered P4 levels [10,18], the upregulation of *cJUN* and *CCNA2* in treated CL appears to result from the presence of some, as yet undefined, compensatory mechanisms in the CL responding to the functional withdrawal of PGs. On the other hand, since the mRNA expression of *cJUN* and *CCNA2* was examined here, it is thus possible that their functional availability is regulated at the protein level or through post-translational modifications, such as phosphorylation [56].

4.5. Conclusions

Interestingly, in our study, most of the effects of Previcox treatment on target gene expression were observed at days 20 and 30 after ovulation. These days fall into the transitional stage between independence and dependence on hypophyseal hormones for CL maintenance [14]. Afterwards, PRL is the main regulator of CL function [58–60]. On the other hand, even though PRL is necessary for CL maintenance, and together with LH its circulating concentrations continuously increase, luteal regression still takes place. At the same time, during progression of the luteal phase, the expression of PRLR in the CL decreases, resembling the course of circulating P4 concentrations [59]. Consequently, in view of our recent findings including the positive effects of PGE2 on PRLR expression in lutein cells [10], and having noted the suppressive effects of Previcox on PRLR expression in the CL, here a new hypothesis is proposed: decreasing support of PGs during the gonadotropin-dependent luteal stage in the dog might lead to lowered PRLR expression and contribute, thereby, to ongoing CL degeneration and diminishing P4 levels.

Thus, indeed, the transition period between the two functional stages of the canine CL appears to be of the utmost importance, marking a change in the regulatory mechanisms of luteal function and the transition between the developing CL, with a high steroidogenic output, and the maintenance luteal stage exhibiting a slow degeneration of the CL accompanied by a progressive decrease in P4 production.

In this context, the results of the present work indicate a modulatory role of PGs that goes beyond the gonadotropin-independent phase, and this despite the observed large variations in expression of several of our target genes. These variations could be due to the limited number of animals available per group and/or individual variations between these animals. On the other hand, however, in particular in the CL of

Table 1
Expression of target genes significantly affected by time. Relative gene expression (RGE) is presented for each control group as geometric mean and geometric standard deviation (± SD). Kruskal–Wallis (non-parametric ANOVA) was applied, followed by Dunn's test. Only comparisons with significant P-value at Dunn's test are represented. $P < 0.05$ was considered statistically significant.

Target	Group	RGE	SD+/SD−	ANOVA p Value	Dunn's test
ETB	Day 0 control	1	10.79/0.92	P = 0.005	d0 vs d5 – P < 0.05 d0 vs d10 – P < 0.05 d0 vs d20 – P < 0.05 d0 vs d30 – P < 0.05
	Day 5 control	26.6	13.82/9.1		
	Day 10 control	42.37	11.28/8.91		
	Day 20 control	35.55	28.66/15.87		
	Day 30 control	17.04	22.34/9.67		
ET2	Day 0 control	5.92	16.57/4.36	P = 0.03	d0 vs d10 – P < 0.05
	Day 5 control	1.66	2.97/1.06		
	Day 10 control	0.99	0.74/0.42		
	Day 20 control	1.88	1.57/0.86		
	Day 30 control	1.86	3.76/1.25		
ECE1	Day 0 control	1	0.77/0.43	P = 0.03	d0 vs d20 – P < 0.01 d0 vs d30 – P < 0.01 d10 vs d20 – P < 0.05 d10 vs d30 – P < 0.05
	Day 5 control	2.04	1.04/0.69		
	Day 10 control	1.45	0.79/0.51		
	Day 20 control	3.17	1.87/1.18		
	Day 30 control	2.88	2.27/1.27		
eNOS	Day 0 control	1	1.07/0.52	P = 0.02	d0 vs d20 – P < 0.05
	Day 5 control	3.1	2.17/1.28		
	Day 10 control	2.16	0.34/0.29		
	Day 20 control	4.61	0.61/0.54		
	Day 30 control	2.94	5.06/1.86		
iNOS	Day 0 control	1	0.91/0.48	P = 0.02	d0 vs d20 – P < 0.05 d0 vs d30 – P < 0.05
	Day 5 control	1.82	1.22/0.73		
	Day 10 control	2.14	0.57/0.45		
	Day 20 control	3.78	2.33/1.44		
	Day 30 control	3.51	1.67/1.13		
ERα/ESR1	Day 0 control	0.99	3.75/0.79	P = 0.01	d0 vs d30 – P < 0.05
	Day 5 control	2.46	1.59/0.97		
	Day 10 control	4.33	4.96/2.31		
	Day 20 control	5.2	1.27/1.02		
	Day 30 control	9.19	4.45/3		
ERβ/ESR2	Day 0 control	1	0.65/0.39	P = 0.001	d10 vs d30 – P < 0.01
	Day 5 control	0.82	0.56/0.33		
	Day 10 control	0.44	0.25/0.16		
	Day 20 control	0.67	0.16/0.13		
	Day 30 control	1.24	0.67/0.44		
YY1	Day 0 control	1	1.4/0.58	P = 0.002	d0 vs d20 – P < 0.01
	Day 5 control	2.39	1.52/0.93		
	Day 10 control	3.2	2.14/1.28		
	Day 20 control	3.43	0.52/0.45		
	Day 30 control	2.92	0.82/1.2		
SF1	Day 0 control	1	1.45/0.59	P = 0.004	d0 vs d20 – P < 0.05 d0 vs d30 – P < 0.05
	Day 5 control	2.11	1.27/0.79		
	Day 10 control	2.59	2.38/1.24		
	Day 20 control	3.08	1.44/0.98		
	Day 30 control	3.03	2.4/1.34		
GATA6	Day 0 control	1.02	0.13/0.12	P = 0.002	d5 vs d20 – P < 0.01 d5 vs d30 – P < 0.05
	Day 5 control	1.14	0.28/0.22		
	Day 10 control	0.91	0.35/0.25		
	Day 20 control	0.68	0.16/0.13		
	Day 30 control	0.61	0.51/0.28		
END	Day 0 control	1	0.58/0.37	P = 0.01	d0 vs d30 – P < 0.01
	Day 5 control	1.7	0.44/0.35		
	Day 10 control	1.16	0.13/0.12		
	Day 20 control	2.21	0.86/0.62		
	Day 30 control	2.32	2.58/1.22		
IL10	Day 0 control	1	2.71/0.73	P = 0.03	d5 vs d30 – P < 0.05
	Day 5 control	3.44	3.97/1.84		
	Day 10 control	2.27	1.5/0.91		
	Day 20 control	0.68	0.18/0.14		
	Day 30 control	0.77	0.1/0.09		

(*continued on next page*)

Table 1 (*continued*)

Target	Group	RGE	SD+/SD−	ANOVA p Value	Dunn's test
TNFα	Day 0 control	1	2.36/0.7	P = 0.008	d5 vs d20 – P < 0.05
	Day 5 control	2.88	1.71/1.08		
	Day 10 control	2.6	3.59/1.51		
	Day 20 control	0.75	1.15/0.46		
	Day 30 control	1.31	0.84/0.51		
TNFR2	Day 0 control	1	1.13/0.53	P = 0.01	d5 vs d30 – P < 0.05
	Day 5 control	2.63	0.87/0.65		
	Day 10 control	1.87	1.02/0.66		
	Day 20 control	1.45	0.54/0.39		
	Day 30 control	0.98	0.27/0.21		

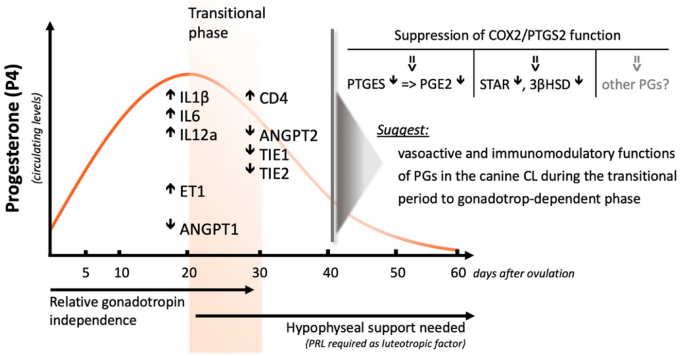

Fig. 4. Schematic representation of effects of functional inhibition of cyclooxygenase 2/prostaglandin synthase 2 (COX2/PTGS2) with Previcox on vascular and immune factors investigated in the present *in vivo* study in the CL of non-pregnant dogs during first 30 days of diestrus. The main findings of the present study are summarized, placing them in perspective with the main features of the canine luteal phase, mainly circulating progesterone (P4) levels and different regulatory phases of the canine CL. Arrows indicate increased (↑) or decreased (↓) expression of different factors in response to inhibition of COX2/PTGS2 activity. When applied in the early luteal phase, Previcox treatment modulates the expression of different factors related to the vascular and immune systems: *ANGPT1*, *ANGPT2*, *TIE1*, *TIE2* (angiopoietin-family, i.e., angiopoietins and their TIE receptors), *ET1* (endothelin 1), interleukins (*IL*)-*1β*, *IL6* and *IL12a* (pro-inflammatory interleukins), *CD4* (cluster of differentiation 4). Together with the previously observed causality between COX2/PTGS2 functionality and luteal PGE2 synthesis by PTGES [10], the decreased expression of several factors involved in regulating CL function in treated dogs suggests a modulatory role of PGs in vascularization and immunity of the canine CL during the transitional phase between independence and dependence on hypophyseal hormones.

Previcox-treated animals, they could possibly also arise from the presence of some compensatory mechanisms, as indicated by the effects of treatment on *cJUN* and *CCNA2* expression. Since the tissue material used for the present study derived from a closed study, we were not able to include additional samples. In the present study, after consulting with the manufacturer for drug safety, double the recommended effective dosage of Previcox was administered to the animals. Despite this higher dosage, pharmacokinetics of firocoxib could reduce its local concentrations at the ovary. This could affect the local effectiveness of the drug, additionally contributing to the obtained variation. Nevertheless, in the context of canine CL physiology, supported by our previous reports [10,59], our observations seem encouraging for further investigation into canine CL regulation.

Finally, supporting our hypothesis, the results presented herein, summarized in Fig. 4, show some potential novel roles of PGs in regulation of the early canine CL, going beyond the P4-stimulatory effects. The involvement of other COX2/PTGS2-derived metabolites on luteal function in the dog remains to be elucidated. Changes induced by inhibition of COX2/PTGS2 in the expression of ANGPT family members (regulators of vascular stability or disruption), seem to indicate a role of PGs in blood vessel stabilization. Also, the increased expression of ET1 in treated animals, as well as of CD4 and pro-inflammatory interleukins, point towards a (immuno)protective role of PGs in the maintenance of CL function.

Having the life span of the CL divided into gonadotropin-dependent and -independent phases, the dog appears to be an interesting model to study mechanisms regulating CL function in a species in which the luteal phase is not affected by possible masking effects of gonadotropins, in particular during early CL development.

Conflict of interests

The authors declare that they have no conflict of interests. All authors read and approved the final version of the manuscript.

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Authors' contribution

MTP was involved in developing the concept of the present study, experimental design, generating data, analysis and interpretation of data and drafting of the manuscript. AG and RN were involved in knowledge transfer, and in the laboratory part of the project, as well as in critical discussion and evaluation of data. TJ was involved in the design of *in vivo* study, tissue collection, knowledge transfer, critical discussion of data and revision of the manuscript. BH was involved in the design of *in vivo* study. BH and AB were involved in knowledge transfer, critical discussion of data and revision of the manuscript. MPK designed and supervised the project, and was involved in interpretation of the data, and drafting and revision of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2019.02.001.

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4.2. Manuscript 2: Global transcriptomic analysis of the canine *corpus luteum* (CL) during the first half of diestrus and changes induced by *in vivo* inhibition of prostaglandin synthase 2 (PTGS2/COX2)

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Miguel Tavares Pereira was involved in developing the concept of the present study, experimental design, generating data, analysis and interpretation of data, and drafting of the manuscript.

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Global Transcriptomic Analysis of the Canine *corpus luteum* (CL) During the First Half of Diestrus and Changes Induced by *in vivo* Inhibition of Prostaglandin Synthase 2 (PTGS2/COX2)

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The canine luteal phase exhibits several peculiarities compared with other species. In early diestrus, the *corpus luteum* (CL) is, at least in part, independent of gonadotropins, and prostaglandins (PGs) appear to be among its main regulators. This was also observed with the inhibition *in vivo* of COX2, when also transcriptional capacity, vascularization and immune-related factors were affected. Here, we aimed to further investigate the potential effects of PGs withdrawal on the CL transcriptome by performing deep RNA sequencing (RNA-Seq). Samples from a previous *in vivo* study were used; bitches were treated for 5, 10, 20, or 30 days after ovulation with firocoxib (Previcox®), a PTGS2/COX2 inhibitor, or a placebo. Analysis of results was performed with SUSHI (framework from FGCZ) and with pathways and functional networks analyzers. Time-dependent effects were also investigated and used for quality control. More highly represented differentially expressed genes (DEGs, $P < 0.01$, FDR < 0.1) in the early CL (days 5 and 10) referred to proliferation and immune system, while in the mature CL (days 20 and 30) they were related with steroidogenesis. The absence of genes concomitantly affected by the treatment at all time-points suggested stage-dependency in the observed effects. Little effect was observed on days 5 and 10. Day 20 had the highest number of DEGs ($n = 1{,}741$), related with increased immune response. On day 30, DEGs found ($n = 552$) referred to decreased steroidogenesis and vascularization. Our results suggest the presence of strong compensatory effects in the early CL and multidirectional effects toward gonadotropin-dependency of the CL after COX2 inhibition.

Keywords: canine (dog), *corpus luteum*, prostaglandins, transcriptome (RNA-seq), diestrus

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INTRODUCTION

The *corpus luteum* (CL) has a central role in pregnancy through its production of progesterone (P4). In dogs, it plays an even more prominent role because it regulates the entire diestric phase. Indeed, the absence of placental steroidogenic activity in this species makes the CL the sole source of pregnancy-supporting P4 (1, 2). Following exceptionally strong preovulatory luteinization (3), the CL continues to develop following ovulation. As in other species, this is supported by the formation of a dense vascular network (4, 5). Serum P4 levels vary greatly among animals and peak between days 15 and 30 after ovulation. Shortly thereafter, luteal function starts to diminish, accompanied by decreasing levels of P4 and signs of cellular degeneration (6–8). The function of the CL is actively terminated in pregnant bitches shortly before parturition (around day 60 after ovulation) during prepartum luteolysis. This is associated with increased circulating prostaglandin (PG)F2α, apparently produced by the fetal placenta (1, 9–12). Interestingly, no such active mechanism is observed in the absence of pregnancy (12, 13). There is no uterine luteolysin in non-pregnant dogs and hysterectomy does not affect CL function (12, 13). Consequently, non-pregnant bitches present a physiological pseudopregnancy, with circulating P4 levels similar to those in pregnant animals (14, 15). It appears thus, that in lacking an active luteolytic principle, the CL life span of non-pregnant dogs is regulated by some intrinsic regulatory mechanisms. At the regulatory level, the canine CL also presents some species-specific peculiarities compared with other domestic animals. Both LH and prolactin (PRL) have luteotropic roles in the canine CL (16–18). However, PRL appears to be the predominant factor and appears to be required for CL maintenance starting around day 25 after ovulation (19, 20). It is noteworthy that ablation of the hypophysis had less effect on CL function in the first 2–4 weeks after ovulation (17). Consequently, the canine CL presents a transitory independence on gonadotropins in its earlier stages, progressing to a gonadotropin-dependent stage at mid-diestrus, with PRL then acting as the predominant luteotropic factor (17, 18, 20).

The initial observation of increased expression of COX2/PTGS2 and PGE2 synthase (PTGES) in early CL stages suggested PGs as possible important regulators of this organ in the dog (21, 22). Further investigations showed direct effects of PGE2 in early canine luteal cells, in which it could increase steroidogenic acute regulatory (STAR) protein expression and P4 production (23). Following these clues, the effects of PGs on CL function were explored *in vivo* (3, 24). For this, firocoxib (Previcox, Merial Ltd.), a specific inhibitor of COX2, was used to treat bitches from the day of ovulation up to 30 days later, with the aim of causing functional withdrawal of PGs (3, 24). In fact, the expression of PTGES and intra-CL levels of PGE2 and PGF2α were significantly decreased by this treatment (3). It also affected the steroidogenic capacity of the CL (decreased expression of 3βHSD and STAR), and suppressed the levels of circulating P4 (3, 24). Furthermore, the decreased nuclear size of luteal cells induced by this treatment was related to their decreased transcriptional capacity (24). These observations showed a causality between COX2 and the PTGES-dependent synthesis of PGE2 in the CL and established PGs as important modulators of CL function (3, 24).

It appears, however, that PGs might have broader effects on the regulation of CL function in addition to steroidogenesis. COX2 inhibition decreased the expression of the PRL receptor (PRLR) while, in parallel, PGE2 could increase the expression of PRLR in isolated canine luteal cells (3). This suggested an indirect role of PGE2, through the enhancement of PRLR expression, to support the luteotropic function of PRL. Based on this, and on the fact that PGE2 could also increase the expression of endothelin receptor B (ETB; important vasodilator) (25), the effects of PGs withdrawal on luteal vascular and immune factors were further investigated (26). The stabilization of blood vessels was negatively affected by the treatment, while the expression of different pro-inflammatory factors was increased (26). Concomitantly, the presence of strong compensatory effects was implied.

Considering the peculiarities of the canine luteal phase, the present study aimed to investigate additional potential effects of withdrawal of PGs on CL physiology. For this, we used samples from a previously reported *in vivo* study (3, 24, 26), and performed a deep RNA sequencing (RNA-Seq) by applying Next Generation Sequencing (NGS) technology. With this approach, we expected to provide deeper understanding of the importance of PGs at different stages of CL development: its early gonadotropin-independence, then its transition to dependence on hypophyseal hormones, and finally the dependence of the mature CL mainly on PRL. Time-dependent changes in the CL transcriptome associated with its development were also investigated in control samples.

MATERIALS AND METHODS

Tissue Samples

This is a follow-up study utilizing tissue material collected in preceding *in vivo* studies (3, 24, 26). Animal experiments were approved by the responsible ethics committee (permit 54/2008) of the University of Warmia and Mazury in Olsztyn, Poland. Briefly, mixed-breed bitches of different ages (2–7 years old) were monitored by vaginal cytology and P4 measurements for the onset of spontaneous estrus. The day when circulating P4 levels exceeded 5 ng/ml was considered the day of ovulation (day 0). Animals were then randomly assigned to four control and four treated groups, receiving orally and daily either a placebo or 10 mg/kg of firocoxib (Previcox, Merial Ltd), respectively. Treatment was maintained for 5, 10, 20 or 30 days after ovulation. Ovaries were collected by ovariohysterectomy on the last day of treatment. *Corpora lutea* were dissected from surrounding ovarian tissue and preserved in RNAlater at −80°C until further use, as described before (23).

RNA Isolation and Purification

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA, following the manufacturer's instructions. A primary evaluation of RNA purity and concentration was performed with a NanoDrop 2000C spectrophotometer (ThermoFisher Scientific AG, Reinach, Switzerland). Further purification of RNA was performed with the RNeasy Mini Kit

(Qiagen GmbH, Hilden, Germany), using the RNA Cleanup protocol provided. RNA integrity was assessed with the Agilent 2200 TapeStation System. RNA integrity numbers (RIN) ranged from 8.0 to 9.8.

RNA-Sequencing and Data Evaluation

Next Generation Sequencing (NGS, RNA-Seq)

Sequencing of RNA with NGS was performed to obtain a quantitative evaluation of gene expression. A total of 32 samples available for this follow-up study were allotted to the following groups: $n = 5$ for day 5 control, $n = 4$ for day 5 treated, $n = 4$ for either day 10 control and day 10 treated, $n = 3$ for either day 20 control and day 20 treated, $n = 4$ for day 30 control and $n = 5$ for day 30 treated. RNA-Seq was performed on $n = 4$ animals/group (control and treated) from days 5, 10, and 30, and $n = 3$ animals/group from day 20 after ovulation. Library preparation, cluster generation and sequencing were performed as previously described (27). All samples were processed at the same time to avoid possible batch effects. In brief, the Qubit (1.0) Fluorometer (Life Technologies, Carlsbad, CA, USA) and Bioanalyzer 2100 (Agilent, Waldbronn, Germany) were used to evaluate the quantity and quality of isolated RNA. To be processed further, samples needed to have a 260/280 nm ratio between 1.8 and 2.1 and a 28S/18S ratio within 1.5–2.0. In the succeeding steps, the TruSeq RNA Sample Prep Kit v2 (Illumina, Inc., San Diego, CA, USA) was used. Total RNA samples (100–1,000 ng) were enriched by poly-A selection and reverse-transcribed to obtain double-stranded cDNA. Additionally, fragmentation, end-repair and polyadenylation steps were performed on cDNA samples before ligation of TruSeq adapters containing the index for multiplexing. PCR was used for selective enrichment of fragments containing TruSeq adapters on both ends, and the quality and quantity of the enriched libraries obtained were assessed with the Qubit (1.0) fluorometer and Caliper GX LabChip GX (Caliper Life Sciences Inc., Hopkinton, MA, USA). Finally, libraries were normalized to 10 nM in Tris-Cl 10 mM with Tween 20.

Cluster generation was performed with 2 nM of pooled normalized libraries on the cBOT System with the TruSeq PE Cluster Kit v4-cBot-HS or TruSeq SR Cluster Kit v4-cBot-HS (Illumina, Inc). Sequencing was performed with the Illumina HiSeq4000 with single end 125 bp using the TruSeq SBS Kit v4-HS (Illumina, Inc.). The raw data (.fastq files) obtained were used for downstream analysis. Additionally, they were also deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE130369.

Data Analysis

The initial analysis of data was performed with the framework SUSHI (28, 29), developed by the Functional Genomics Center of Zurich (FGCZ ETH/UZH, Zurich, Switzerland). Spliced Transcripts Alignment to a Reference (STAR) software was used to align the RNA-Seq dataset (30) to a reference canine genome, the Ensembl genome build CanFam3.1 (http://www.ensembl.org/Canis_familiaris/Info/Index). Gene expression values were obtained with the function *featureCounts* from the R package Rsubread (31). A minimum average of 10 reads in at least one group of replicates was required to consider a gene as detected. After reads counting, initial quality control and explorative analysis were performed with the SUSHI framework.

For differential expression analysis different contrasts (pairwise comparisons) were defined and gene differential expression for each contrast was assessed with the generalized linear model approach from the Bioconductor package DESeq2 (32). This was performed as previously described (27), using the Wald test to assess the significance of differential expression. Next, correction of multiple testing was obtained with the Benjamini-Hochberg algorithm that computes False Discovery Rate (FDR, adjusted *P*-value). Finally, thresholds of *P*-value $<$ 0.01 and adjusted *P*-value $<$ 0.1 (i.e., FDR $<$ 10%) were applied. The complete differentially expressed genes (DEGs) lists obtained for each contrast were used for downstream analyses and are provided in **Supplemental Table 1** (for time-dependent effects) and **Supplemental Table 2** (for treatment-induced effects).

Functional characterization of DEGs for each contrast was performed by identifying different functional terms, resorting to available bioinformatics resources. The identification of over-represented functional categories (i.e., gene ontologies) and their enrichment scores were calculated using the web-based Panther software [http://pantherdb.org, (33)]. This analysis was further supported and complemented with the web-based software Enrichr [http://amp.pharm.mssm.edu/Enrichr/, (34)]. The identification and visualization of enriched functional biological networks was obtained with the application ClueGO v2.5.1 (35) for the software platform Cytoscape v3.6 (36). The software Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA, USA) was used to predict the most significantly affected canonical pathways and identify possibly involved upstream regulators. Lists of up to 10 representative genes involved with particular functional terms (gene ontologies, functional networks, canonical pathways and upstream regulators) from each contrast are presented in **Supplemental Table 3**. In addition, Venn diagram was generated to identify DEGs concomitantly affected by treatment in different contrasts. For this analysis, the thresholds were defined for *P*-value $<$ 0.01 and fold-change for up- (log2Ratio $\geq$ 1) and down-regulation (log2Ratio $<$ -1). Full lists of DEGs used as input for Venn diagram and genes present on each intersection are provided in **Supplemental Table 4**.

Expression of Selected Target Genes by Semi-quantitative Real-Time TaqMan qPCR

Further validation of the RNA-Seq data obtained and analysis of specific functional pathways suggested by our analysis were performed through evaluation of the mRNA expression of 20 selected candidate target genes, by semi-quantitative real-time (TaqMan) qPCR. All available samples were used for qPCR experiments ($n = 32$). A complete list of primers used, TaqMan probes and pre-designed (i.e., commercially-available) TaqMan systems is presented in **Table 1**. Self-designed primers and 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (TAMRA) labeled probes were designed and ordered from Microsynth AG (Balgach,

TABLE 1 | List of gene symbols, corresponding gene names and TaqMan systems used for real time qPCR.

Gene	Name	Accession number	Primer sequence		Product length (bp)
IDO1	Idoleamine 2,3-dioxygenase 1	XM_532793.5	Forward	5′-TGA TGG CCT TAG TGG ACA CAA G-3′	116
			Reverse	5′-TCT GTG GCA AGA CCT TTC GA-3′	
			TaqMan probe	5′-CAG CGC CTT GCA CGT CTG GC-3′	
SULT1E1	Sulfotransferase family 1E	MK728829	Forward	5′-AAC AGA TGG CAT CTC CTA GAG TAG TG-3′	100
	member 1		Reverse	5′-CGG CAA AGA TAG ATC ACC TTA CAG T-3′	
			TaqMan probe	5′-CCA TCT GCC AGT TGA ACT TCT TCC AGC C-3′	
TBXAS1	Thromboxane A synthase 1	XM_005629559.1	Applied Biosystems, prod. no. Cf01022701_m1		105
PTGDS	Prostaglandin D synthase	NM_001003131.1	Applied Biosystems, prod. no. Cf02622002_m1		85
TGFβ1	Transforming growth factor beta 1	NM_001003309.1	Applied Biosystems, prod. no. Cf02623324_m1		83
TGFβR1	Transforming growth factor beta receptor 1	XM_014117881.1	Applied Biosystems, prod. no. Cf02687913_m1		110
ICAM1	Intercellular adhesion molecule 1	NM_001003291.1	Applied Biosystems, prod. no. Cf02690470_u1		124
NODAL	Nodal growth differentiation factor	XM_546146.3	Applied Biosystems, prod. no. Cf02711306_u1		149
FAS	Fas cell surface death receptor	XM_005636650.1	Applied Biosystems, prod. no. Cf02651136_m1		118
FASLG	Fas ligand	NM_001287153.1	Applied Biosystems, prod. no. Cf02625215_s1		89
NFκB1	Nuclear factor kappa B subunit 1	NM_001003344.1	Applied Biosystems, prod. no. Cf02689968_m1		119
NFκBIa	NFKB inhibitor alpha	XM_537413.5	Applied Biosystems, prod. no. Cf02741714_m1		129
PDGF B	Platelet derived growth factor subunit B	NM_001003383.1	Applied Biosystems, prod. no. Cf02626637_m1		109
FGF1	Fibroblast growth factor 1	XM_014108102.1	Applied Biosystems, prod. no. Cf02716346_g1		77
FGF2	Fibroblast growth factor 2	XM_003432481.3	Applied Biosystems, prod. no. Cf03460065_g1		147
THBS1	Thrombospondin 1	XM_544610.5	Applied Biosystems, prod. no. Cf02701399_m1		88
PPARγ	Peroxisome proliferator activated receptor gamma	NM_001024632.2	Applied Biosystems, prod. no. Cf02625640_m1		92
EGLN1	PHD2/egl-9 family hypoxia inducible factor 1	XM_546089.4	Applied Biosystems, prod. no. Cf02713521_m1		115
NR4A1	Nuclear receptor subfamily 4 group A member 1	NM_001003227.1	Applied Biosystems, prod. no. Cf02719047_s1		113
HSD17B7	Hydroxysteroid 17β dehydrogenase 7	XM_014111264.1	Applied Biosystems, prod. no. Cf02657821_m1		82
PTK2	Protein tirosine kinase 2	XM_005627993.2	Applied Biosystems, prod. no. Cf02684608_m1		104
EIF4H	Eukaryotic translation initiation factor 4H	XM_014114129.1	Applied Biosystems, prod. no. Cf02713640_m1		136
KDM4	Lysine (K)-specific demethylase 4A	XM_005629106.2	Applied Biosystems, prod. no. Cf02708629_m1		96

Switzerland). The design of *IDO1* primers and probes was based on published canine CDS sequences while for *SULT1E1* molecular cloning of the canine sequence was required and this was performed by a subcloning approach using the pGEM-T vector, as described previously (22, 37, 38) (sequence submitted to GenBank under accession number MK728829). Commercially-available systems were obtained from Applied Biosystems. The efficiency of PCR reactions was evaluated to ensure approximately 100% (39).

Elimination of genomic DNA contamination, reverse transcription (RT) and semi-quantitative real-time TaqMan qPCR (RT-qPCR) were performed following the manufacturers' and our previously published protocols (22, 39). Briefly, total RNA samples were treated with the RQ1 RNase-free DNase kit (Promega, Dübendorf, Switzerland) to eliminate possible genomic DNA contamination. Random hexamers were used as primers in the subsequent RT reactions, employing reagents obtained from Applied Biosystems. For RT-qPCR, all samples were run in duplicates in 96-well optical plates, using autoclaved water and RNA not subjected to RT (minus-RT control) instead of cDNA as negative controls. Reactions were run in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems) and were set as follows: initial denaturation for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C each. Relative quantification was done by applying the comparative Ct method (ΔΔCt). RT-qPCR data were normalized with three reference genes: PTK2, EIF4H, and KDM4. Based on the RNA-Seq data collected herein, these genes were found to be stably expressed in all samples examined using the online tool RefFinder and NormFinder software (40, 41). Since RT-qPCR data were unevenly distributed, logarithmic transformation was performed and results are presented as geometric means (Xg) ± geometric standard deviation (SD). The evaluation of treatment-induced effects was performed with

a two-tailed Student's *t*-test, comparing the treatment group with the control group at each time point (day). Additionally, time-related changes in the expression of target genes were evaluated in control animals using the Kruskal-Wallis test (non-parametric ANOVA) followed by Dunn's test. All statistical tests were performed with GraphPad 3 (GraphPad Software Inc., San Diego, CA, USA) and values of $P < 0.05$ were considered statistically significant.

RESULTS

Initial Evaluation of Sequencing Results

Initial exploratory analysis of the sequencing data obtained was performed with the CountQC app provided in the SUSHI framework. This function allowed assessment of homogeneity and correlations between samples, as well as clustering of high variance features among all samples submitted for RNA-Seq. The analysis of samples correlation matrix (**Supplemental Image 1A**) indicated the presence of some variation among all sequenced samples. Based on the analysis of this matrix and gene expression scatter plots (not shown), samples 10/1 and 30/1 controls, and 10/2 and 30/1 treated with Previcox, were considered outliers and removed from the dataset for further analysis. This allowed a more homogeneous correlation between samples within each group (**Supplemental Image 1B**). Further observation of the correlation matrix indicated a higher homogeneity among samples of each control group compared with the respective treated groups (**Supplemental Image 1B**). This was also suggested by the more homogeneous clustering of samples and genes visible on the heatmap, with 2,000 genes exhibiting higher variance among control samples (**Supplemental Image 1C**) compared with the one that contained all analyzed samples (**Supplemental Image 1D**). Clustering observed on the heatmap with the 2,000 genes showing higher variance among all analyzed samples (**Supplemental Image 1D**) also suggested that, apart from effects on the immune system and negative regulation of cellular processes on day 20, passage of time appeared to have a higher global impact on CL expression of these genes than COX2 inhibition. This was further visualized using a principal component analysis (PCA) plot of the same 2,000 genes with higher variance among all samples (**Figure 1**). Thus, samples appeared to be segregated based on time-point of analysis, with those samples from days 5 and 10 being proximally distributed on a cluster and samples from days 20 and 30 on the other side of the plot. The scattered distribution of samples treated up to day 20 after ovulation indicates stronger effects of treatment in this group (**Figure 1**).

Time-Dependent Effects

Samples available for the present study were representative of different regulatory stages of early CL development, i.e., the early developing, gonadotropin-independent CL (days 5 and 10), and the mature CL in transition and during its dependence on gonadotropins (days 20 and 30, respectively). Close similarity between samples from days 5 and 10 on the one end, and days 20 and 30 on the other end, was also suggested from the PCA plot (**Figure 1**). Therefore, when evaluating the effects of time on the CL transcriptome, samples were grouped accordingly with differential expression analysis (pairwise comparison) being performed for the contrast "Days 20+30 control over days 5+10 control." This analysis was performed using the DESeq2 package for Bioconductor and genes were assumed to be differentially expressed if $P < 0.01$ and FDR < 0.1. Of the total set of 19,856 genes, 13,332 genes were considered expressed (above the threshold of 10 reads per gene). DEGs accounted for 3,484 features with 1,681 being up- and 1,803 down-regulated at days 20+30 compared with the early developing CL (days 5+10; **Table 2**, **Figure 2A**). A detailed list of all DEGs affected by time is provided in **Supplemental Table 1**. Additional representation of DEGs in Volcano plots, filtered by FDR < 0.1 and log2Ratio > 0.5, is provided in **Supplemental Image 2A**. Following this, functional characterization of DEGs was performed. First, the pairwise comparison was classified according to Gene Ontology (GO) terms related to the domain biological process (BP). Panther software was used to calculate enrichment scores for each term, and results were further corroborated with Enrichr. Lists of up to ten (n = 10) representative genes for different functional terms, as well as upstream regulators and networks identified in the following steps, are presented in **Supplemental Table 3**.

Genes more highly represented at early CL stages (days 5+10) compared to days 20+30 were strongly associated with (**Figure 2A**, **Supplemental Table 3**): cell-cell adhesion ($P =$ 2.40E-3), locomotion ($P =$ 1.28E-3), immune system process ($P =$ 1.27E-3), metabolic process ($P =$ 9.67E-4) and cellular process ($P =$ 2.73E-4). On the other hand, functional terms over-represented in the mature CL (days 20+30) included (**Figure 2A**, **Supplemental Table 3**): fatty acid biosynthetic process ($P =$ 1.26E-3), fatty acid metabolic process ($P =$ 2.96E-4), lipid metabolic process ($P =$ 1.12E-6), phospholipid metabolic process ($P =$ 2.35E-3), protein localization ($P =$ 1.64E-3), intracellular protein transport ($P =$ 1.04E-3), transport ($P =$ 3.12E-5) and localization ($P =$ 1.22E-4).

Using as input the lists of upregulated and downregulated DEGs for this contrast (**Supplemental Table 1**), enriched functional networks were grouped and visualized with the ClueGO plug-in for the platform Cytoscape (**Figure 2B**, **Supplemental Table 3**, **Supplemental Image 3A**). Among the more highly represented functional networks observed on days 5 and 10 were those referring to cell signaling and metabolism, extracellular matrix, apoptosis and, in greater numbers, networks related to immune function (**Figure 2B**, **Supplemental Table 3**). On the other hand, networks mainly observed on days 20 and 30 were associated with intracellular transport and (lipids) metabolism (**Supplemental Table 3**, **Supplemental Image 3A**).

The prediction of the most significantly affected signaling pathways was obtained from IPA software by using the list of DEGs as input ($P < 0.01$, FDR < 0.1). Among the most enriched canonical pathways predicted to be activated by the passage of time were those related to (**Supplemental Table 3**): cholesterol synthesis/steroidogenesis (superpathway of cholesterol biosynthesis, $P =$ 4.90E-5; cholesterol biosynthesis I, $P =$ 1.20E-4; cholesterol biosynthesis II, $P =$ 1.20E-4; and cholesterol biosynthesis III, $P =$ 1.20E-4). On the other hand, among

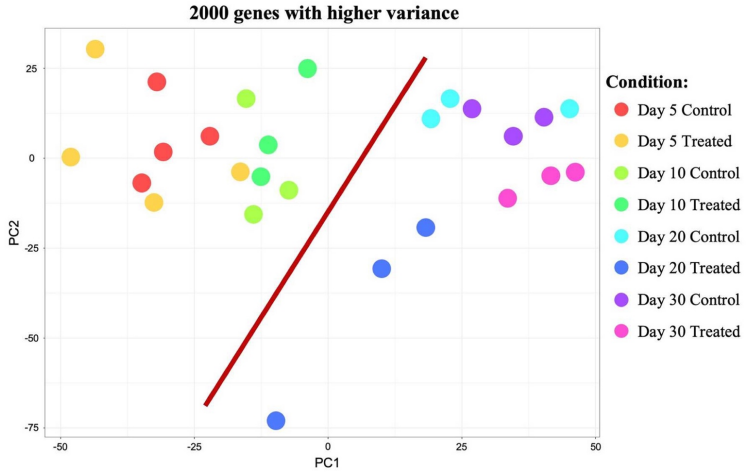

FIGURE 1 | Principal component analysis (PCA) plot of all samples showing the 2,000 genes that presented higher variance. Distribution of samples appears to be mainly based on the effect of time (highlighted by red bar separating samples from days 5 and 10 from samples from days 20 and 30). Samples distribution and scattering also suggest stronger effects of treatment on day 20 than on the other time points studied.

TABLE 2 | Summary of differential expression analysis (pairwise comparison) for all selected contrasts investigated in the present study.

Contrasts	Days 20+30 C over days 5+10 C	Day 5 T over day 5 C	Day 10 T over day 10 C	Day 20 T over day 20 C	Day 30 T over day 30 C
Total DEGs ($P < 0.01$, FDR < 0.1)	3,484	74	2	1,741	552
Number of genes with counts above threshold (10 reads per gene)	13,332	13,428	13,280	13,477	13,187
Upregulated genes	1,681	47	1	1,146	306
Downregulated genes	1,803	27	1	595	246

significant pathways that were predicted to be inhibited by time were those related to (**Supplemental Table 3**): cellular proliferation (EIF2 signaling, $P = 7.94E-16$; mTOR signaling, $P = 3.98E-12$), cell cycle (cyclins and cell cycle regulation, $P = 3.63E-7$), ECM production (inhibition of matrix metalloproteases, $P = 1.86E-4$) and immune function (leukocyte extravasation signaling, $P = 3.55E-8$; acute phase response signaling, $P = 5.89E-6$; IL8 signaling, $P = 3.63E-5$; chemokine signaling, 4.68E-5; dendritic cell maturation, $P = 1.8E-4$).

The analysis of DEGs with IPA allowed additional identification of upstream regulators possibly affecting expression of the DEGs obtained. Among the predicted upstream regulators, the following factors were identified: transforming growth factor β 1 (TGFβ1, $P = 7.27E-33$), tumor necrosis factor (TNF, $P = 5.88E-26$), beta-estradiol ($P = 3.08E-22$), platelet-derived growth factor BB (PDGF BB, $P = 1.08E-18$), interferon gamma (INFγ, $P = 3.47E-15$), progesterone (P4, $P = 3.86E-15$), insulin growth factor 1 (IGF1, $P = 3.85E-13$), nuclear factor kappa B inhibitor alpha (NFκBIα, $P = 5.86E-13$) and prostaglandin (PG) E2 receptor 2 (PTGER2/EP2, $P = 9.5E-12$) (**Supplemental Table 3**).

Treatment-Induced Effects

Differential Expression Analysis (Pairwise Comparison) and Venn Diagram

The effects of treatment with Previcox on the transcriptome of CL tissue were assessed by differential expression analysis, in which treated samples were compared with control samples at the respective time-points (days 5, 10, 20 and 30). Thus, the following contrasts were defined: "day 5 treated over day 5 control," "day 10 treated over day 10 control," "day 20 treated over day 20 control," and "day 30 treated over day 30 control." As described for time-dependent effects, the DESeq2 package for Bioconductor was used to obtain lists of DEGs. The thresholds of $P < 0.01$ and FDR < 0.1 were applied to consider a gene as being differently expressed. For all contrasts, a total of 19,856 genes were identified; only genes with at least 10 reads were considered as expressed and included in further analyses. A summary of

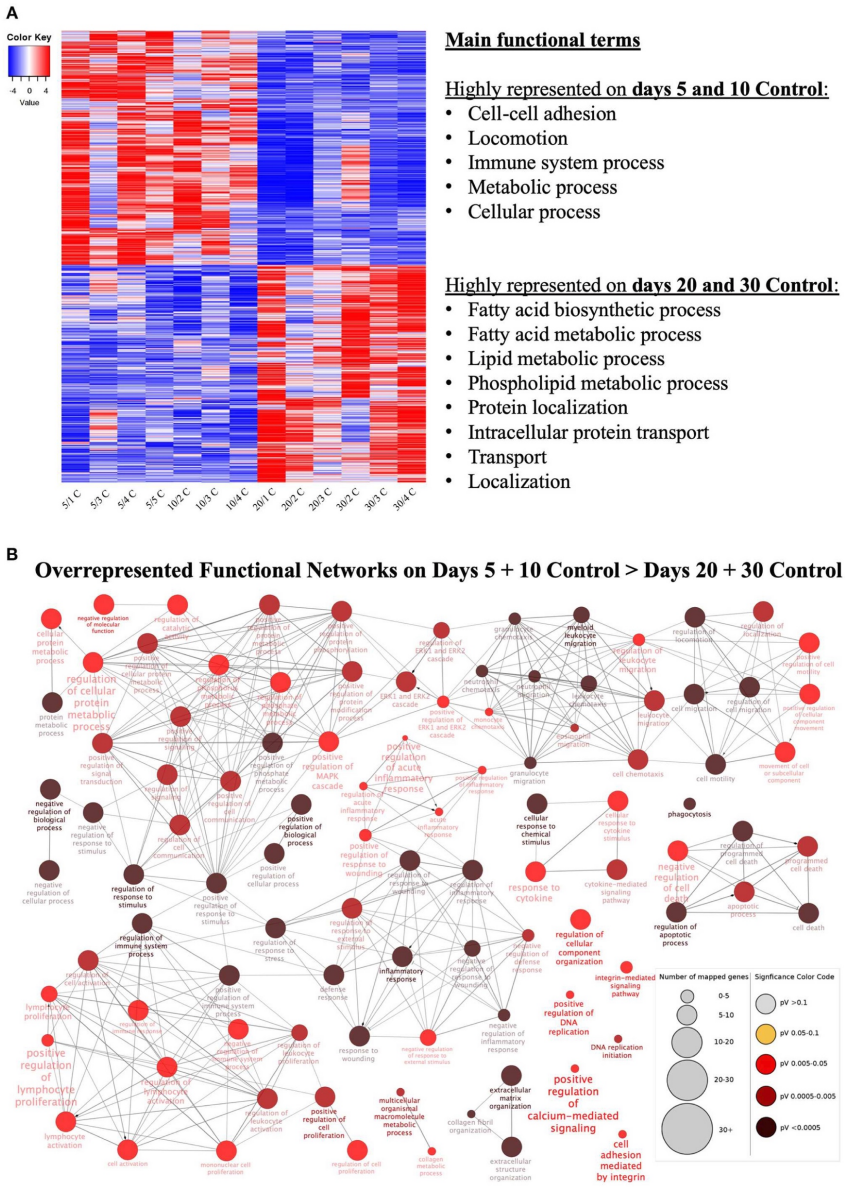

FIGURE 2 | Functional categorization of differentially expressed genes (DEGs) affected at selected time points (contrast "days 20+30 control over days 5+10 control"). **(A)** Heatmap of 3,484 DEGs in the contrast "days 20+30 over days 5+10 control." Gradient of high to low expression of each gene relative to average expression is indicated by red to blue colors. 1,803 genes were more highly expressed in early (days 5 and 10) CL stages whereas 1,681 genes were more highly expressed in mid-diestrus (days 20 and 30) stages ($P \leq 0.01$, FDR ≤ 0.1). Representative overrepresented functional terms in each group of upregulated genes are listed (statistical details are provided in the text and **Supplemental Table 3**). Entire list of DEGs is provided as **Supplemental Table 1**. **(B)** Functional networks found for downregulated DEGs from the contrast "days 20+30 control over days 5+10 control." Overrepresented functional terms of days "5+10 control" are shown. Redundant or non-informative terms were removed and the networks obtained were manually rearranged. Number of mapped genes is indicated by the node size, while significance of functional terms is denoted by node color (represented in legend at the right bottom corner). Networks more highly represented on days 5 and 10 control (developing gonadotropin-independent CL) were related to immune function, extra-cellular matrix (ECM), intracellular signaling and apoptosis.

DEGs analysis is presented in **Table 2**, while full lists of DEGs in response to all treatments at every time-point are presented in **Supplemental Table 2**.

For the contrast at day 5, after being filtered ($P < 0.01$, FDR < 0.1), 74 genes were considered to be DEGs, of which 47 were upregulated and 27 downregulated after treatment. As for the contrast "day 10 treated over day 10 control," the expression of genes varied greatly individually, and resulted in only 2 DEGs filtered by the applied *P*-value/FDR thresholds. Following this low number of DEGs found at day 10, no functional characterization could be performed for this contrast. In the contrast for day 20, 1,741 genes met the criteria of $P < 0.01$ and FDR < 0.1, making this the treatment-related contrast with the highest number of DEGs. Of these DEGs, 1,146 were more highly expressed in the day 20 treated group, while 595 were more highly expressed in the day 20 control group. Finally, for the contrast "day 30 treated over day 30 control" 552 genes were differently expressed ($P < 0.01$, FDR < 0.1), comprising 306 up- and 246 down-regulated features in response to prostaglandin withdrawal. Further representation of DEGs (FDR < 0.1; log2ratio > 0.5) for the contrasts "day 5 treated over day 5 control," "day 20 treated over day 20 control," and "day 30 treated over day 30 control" in form of Volcano Plots are shown in **Supplemental Image 2B–D**.

In an attempt to identify genes that would be simultaneously affected by COX2-inhibition at different time-points of the CL life span, the intersections of DEGs from the contrasts at days 5, 20, and 30 were visualized with a Venn diagram (**Figure 3A**). The input DEGs were filtered for $P < 0.01$ and log2Ratio < -1 (downregulated) and log2Ratio > 1 (upregulated), and a complete list of genes from each intersection is provided in **Supplemental Table 4**. Thirty-two genes were simultaneously affected by treatment on days 5 and 20, while 78 were shared among days 20 and 30. Despite the time difference, 4 genes were still found to be simultaneously affected on days 5 and 30. However, no gene was concomitantly affected by prostaglandin withdrawal at the different time-points.

Functional Annotations, Networks, and Pathways

Further characterization of DEGs for each contrast was performed by identifying different enriched functional terms. The analysis flow was similar to the one applied in differential expression analysis of time-related effects: functional terms (GOs) were identified with Panther and Enrichr, functional networks were visualized with Cytoscape, and prediction of affected canonical pathways and upstream regulators involved was done by IPA. For input, DEGs were filtered by $P < 0.01$ and FDR < 0.1; lists of representative genes are provided in **Supplemental Table 3**.

Contrast "day 5 treated over day 5 control"

The main functional terms related to genes more highly expressed in treated samples from day 5 were (**Figure 3B**, **Supplemental Table 3**): positive regulation of intracellular transport ($P = 3.48E-8$), regulation of cell motility ($P = 1.31E-6$), regulation of cell migration ($P = 7.40E-6$), regulation of cellular component movement ($P = 2.59E-6$) and regulation of locomotion ($P = 2.84E-6$). Due to low numbers of input DEGs, no significant gene ontologies could be identified among the DEGs downregulated by treatment at this early gonadotropin-independent luteal phase. This also accounts for the Cytoscape analysis of functional networks from up- and down-regulated genes.

Enriched pathways predicted to be activated by IPA were mostly related to (**Supplemental Table 3**): cytoskeleton/cell movement/cell division (RhoA signaling, $P = 1.10E-6$; actin cytoskeleton signaling, $P = 4.07E-5$; signaling by Rho family GTPases, $P = 6.31E-5$; regulation of actin-based motility by Rho, $P = 1.07E-4$). Additionally, the pathways death receptor signaling ($P = 5.50E-6$) and leukocyte extravasation signaling ($P = 2.75E-4$) were also predicted to be activated by treatment, while the RhoGDI signaling ($P = 5.13E-7$) pathway was predicted to be deactivated. Among the predicted upstream regulators, actin alpha cardiac muscle 1 (ACTC1, $P = 7.59E-6$), PDGF BB ($P = 3.13E-5$), actin alpha 2 (ACTA2, $P = 2.1E-4$), TNF ($P = 2.78E-4$), and TGFβ1 ($P = 9.94E-E4$) were found (**Supplemental Table 3**).

Contrast "day 20 treated over day 20 control"

For this contrast, the following main terms more highly represented in the treated mature CL during its transition to gonadotropin dependence on day 20 were found (**Figure 3C**, **Supplemental Table 3**): cell proliferation ($P = 4.78E-4$), regulation of cell cycle ($P = 7.06E-5$), cell-cell adhesion ($P = 9.85E-4$), locomotion ($P = 5.58E-5$), response to external stimulus ($P = 1.20E-3$), cell differentiation ($P = 1.84E-4$), regulation of phosphate metabolic process ($P = 2.03E-4$), developmental process ($P = 1.93E-5$), cellular component organization or biogenesis ($P = 6.12E-4$) and metabolic process ($P = 1.46E-3$). High functional variation was found for DEGs in control samples on day 20, yielding only low enrichment scores without strongly enriched functional terms, and without any strongly enriched functional networks. This differed from the CL samples derived from dogs treated for 20 days with Previcox, in which strongly over-represented networks were found, referring to cell differentiation, cell death, gene expression, translation and signaling, hormone regulation and immune function (**Figure 4**, **Supplemental Table 3**).

In response to Previcox treatment, the significant canonical pathways that were predicted to be activated (**Supplemental Table 3**) were related by IPA analysis to cellular proliferation/growth (EIF2 signaling, $P = 5.01E-21$; mTOR signaling, $P = 1.86E-9$) and immune function (toll-like receptor signaling, $P = 1.66E-4$; adrenomedulin signaling pathway, $P = 1.20E-3$; and acute phase response signaling, $P = 4.37E-3$; NFκB signaling, $P = 4.47E-3$; IL6 signaling, $P = 4.47E-3$). Additionally, the relaxin signaling ($P = 4.68E-4$) pathway was also predicted to be activated while the angiopoietin signaling ($P = 4.47E-3$) pathway was predicted to be deactivated. The list of top upstream regulators for the observed effects included (**Supplemental Table 3**): β-estradiol ($P = 6.66E-23$), PDGF BB ($P = 9.63E-20$), TGFβ1 ($P = 2.26E-15$), IL1β ($P = 1.1E-14$), TNF ($P = 4.27E-14$), PGE2 ($P = 3.68E-11$), NFκBIA ($P = 1.45E-10$),

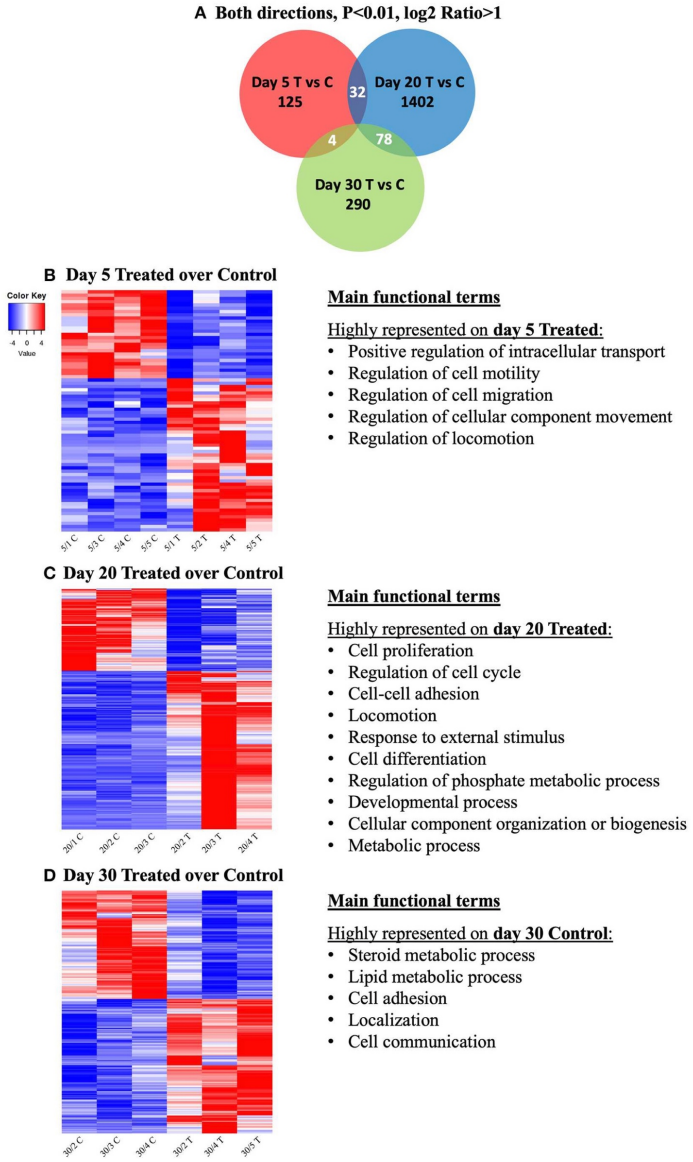

FIGURE 3 | Venn diagram showing the distribution and overlap of differentially expressed genes (DEGs) induced by treatment at different time-points **(A)**. Lists of DEGs from the contrasts "day 5 treated over day 5 control," "day 20 treated over day 20 control," and "day 30 treated over day 30 control" were used as input data. Thresholds were defined for *P*-value < 0.01 and fold-change for up (log2Ratio ≥ 1) and downregulated (log2Ratio ≤ -1) genes. No gene was found to be concomitantly affected by treatment in all groups. Complete list of genes from each contrast and intersection are present in **Supplemental Table 4**. Heatmap and overrepresented functional terms present in differentially expressed genes (DEGs) induced by treatment at different studied time-points **(B–D)**. Gradient of high to low expression of each gene relative to average expression is indicated by red to blue colors. Main significant overrepresented functional terms (gene ontologies) in each group of upregulated genes are listed (statistical details are provided in the text and **Supplemental Table 3**). Entire list of DEGs is provided as **Supplemental Table 2**. **(B)** Heatmap of 74 DEGs of the contrast "day 5 treated over day 5 control" and representative significant overrepresented functional terms in "day 5 treated" are shown. **(C)** Heatmap of 1,741 DEGs of the contrast "day 20 treated over day 20 control" and representative significant overrepresented functional terms at "day 20 treated". **(D)** Heatmap of 552 DEGs of the contrast "day 30 treated over day 30 control" and representative overrepresented functional terms in "day 30 control".

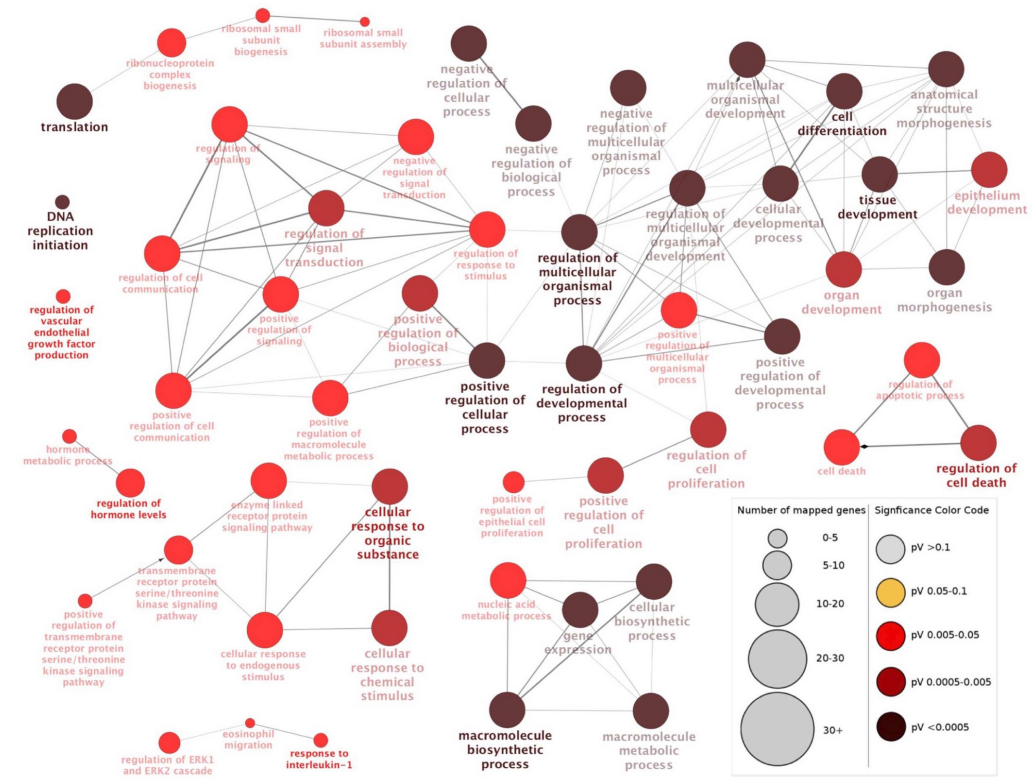

FIGURE 4 | Functional networks found in upregulated differentially expressed genes (DEGs) from the contrast "day 20 treated over day 20 control." Overrepresented functional terms of "day 20 treated" are shown. Redundant or non-informative terms were removed and the networks obtained were manually rearranged. Number of mapped genes is indicated by the node size while significance of functional terms is denoted by node color (represented in legend at the right bottom corner). Networks more highly represented in CL samples from animals treated until day 20 after ovulation were related to gene expression and translation, signaling, hormone regulation, cell differentiation and death, and immune function.

Fas cell surface death receptor (FAS, P = 1.89E-9) and P4 (P = 2.14E-9).

Contrast "day 30 treated over day 30 control"

Genes higher expressed in day 30 control samples were related with the following functional terms (**Figure 3D**, **Supplemental Table 3**): steroid metabolic process (P = 2.32E-4), lipid metabolic process (P = 1.07E-4), cell adhesion (P = 1.33E-3), localization (P = 1.82E-3) and cell communication (P = 8.96E-4). No significantly enriched GO and networks could be found for genes upregulated in response to treatment on day 30, which was restricted by higher functional variation of identified DEGs. However, in control samples functional networks related to nitric oxide synthesis and angiogenesis were found to be highly enriched (**Supplemental Table 3**, **Supplemental Image 3B**).

Interestingly, canonical pathways predicted to be deactivated by treatment in mature CL at day 30 were related with (**Supplemental Table 3**): cholesterol synthesis/steroidogenesis (superpathway of cholesterol biosynthesis, P = 1.35E-7; cholesterol biosynthesis I, P = 6.31E-6; cholesterol biosynthesis II, P = 6.31E-6; cholesterol biosynthesis III, P = 6.31E-6), immune signaling (acute phase response signaling, P = 1.91E-4; NFκB signaling, P = 3.47E-4; TGFβ signaling, P = 5.13E-3; IL8 signaling, P = 6.61E-3; leukocyte extravasation signaling, P = 8.71E-3) and vascularization (VEGF signaling, P = 3.31E-3; PDGF signaling, P = 1.48E-3). With regard to cellular proliferation, the EIF2 signaling (P = 3.16E-13) pathway was predicted to be activated, while the mTOR signaling (P = 2.63E-7) pathway was predicted to be deactivated. Among the top upstream regulators predicted with IPA software were

(**Supplemental Table 3**): TNF (P = 1.89E-10), peroxisome proliferator-activated receptor gamma (PPARγ, P = 9.22E-5), TGFβ1 (P = 9.58E-5), fibroblast growth factor 2 (FGF2, P = 1.44E-4) and NFκBIA (P = 2.25E-4).

Expression of Candidate Target Genes

The expression of candidate genes was investigated by semi-quantitative (TaqMan) qPCR in 32 samples from the different available groups. All results for time-dependent and treatment effects are cumulatively presented in **Supplemental Table 5**. Extracted, significant results were prepared for the main document and are shown in **Tables 3**, **4**. The functional groups chosen for validation of transcriptional analysis included: eicosanoid synthases (*TBXAS1*, *PTGDS*), immune factors (*TGFβ1*, *TGFβR1*, *ICAM1*, *IDO1*, *NODAL*, *FAS*, *FASLG*, *NFκB1*, *NFκBIA*), growth factors (*PDGF B*, *FGF1*, *FGF2*), vascular regulators (*THBS1*), hypoxia-related factors (*EGLN1*/PHD2), nuclear receptors (*PPARG*, *NR4A1*) and steroid-related factors (*HSD17B7*, *SULT1E1*). Expression of all candidate genes was detected in all tissues and, generally, a good correlation was found between RNA-Seq and RT-qPCR results. Additionally, no significant changes in the expression of *EGLN1* and *PDGF B* could be found in response to treatment or passage of time.

Time-Dependent Effects

Changes in candidate gene expression associated with time were assessed in control samples. All significant effects observed with the passage of time (including statistical analysis) are presented in **Table 3**. Time-related effects were observed for: *TBXAS1*(P = 0.0006), *PTGDS* (P = 0.0153), *TGFβR1* (0.0017), *ICAM1* (P = 0.0003), *FAS* (P = 0.0015), *FASLG* (P < 0.0001), *NFκBIA* (P = 0.0006), *FGF1* (0.0031), *FGF2* (P = 0.0032), *THBS1* (P < 0.0001), *PPARγ* (P = 0.0183) and *HSD17B7* (P < 0.0001) (**Table 3**, **Supplemental Table 5**). Despite that P < 0.05 was obtained for *NFκB* (P = 0.0408) with the ANOVA test, no significant effect was obtained in the multiple comparisons test (P > 0.05, **Table 3**, **Supplemental Table 5**).

The expression of most target genes was downregulated with the passage of time. Expression of *TBXAS1*, *ICAM1*, *FAS*, *FASLG*, and *THBS1* was significantly higher in early developing CL stages (days 5 and/or 10) and decreased toward CL maturation (days 20 and/or 30). The expression of *PTGDS*, *NFκBIA*, and *PPARγ* was significantly higher on day 10 than on day 20. *TGFβR1* and *FGF2* expression was the lowest on day 30 compared with days 5 and 20 or only with day 20, respectively. In a different direction, the expression of *FGF1* significantly increased from day 5 to day 30. Finally, *HSD17B7* had the lowest expression at day 5, increasing during later stages of CL development.

Treatment-Induced Effects

Effects of Previcox treatment were assessed in all available samples by comparing expression of candidate genes in treated and control groups at each time-point (days 5, 10, 20, and 30). No significant changes (P > 0.05) in the expression of *FGF1* and *HSD17B7* were obtained in response to treatment in any of the comparisons studied (**Supplemental Table 5**), even if their expression was predicted to be modulated by NGS on days 30 and 20. Additionally, no significant changes in the expression of any of the candidate genes could be observed on day 10 (**Supplemental Table 5**). In the pairwise comparison on day 5, *TGFβ1* and *THBS1* exhibited increased expression in response to treatment, while *FASLG*, *FGF2* and *SULT1E1* were downregulated (for details, including statistical analysis, see **Table 4A**). At day 20, higher expression of *TGFβR1* and *FGF2* was observed in control samples (**Table 4B**). In a different direction, several factors were upregulated by treatment on day 20: *TBXAS1*, *PTGDS*, *ICAM1*, *NODAL*, *FAS*, *FASLG*, *NFκB1*, *NFκBIA*, *THBS1*, *PPARG*, *NR4A1*, and *SULT1E1* (**Table 4B**). Finally, on day 30, treatment decreased the expression of *IDO1* and increased the expression of *FGF2* (**Table 4C**).

DISCUSSION

General Considerations

Among many species-specific regulatory features, the presence of a transitional gonadotropin independence in the developing canine CL is certainly one of the most intriguing (17, 20). It positions the dog as a valuable model for investigating CL development without the dominant effects of hypophyseal hormones that are observed, e.g., in livestock (42, 43). As described previously, PGs, mainly PGE2, are considered to be among the most important regulators of CL function during its independence from gonadotropins (3, 23, 24). It also became apparent that PGE2 might have a broader role in the canine CL, regulating luteal sensitivity to other hormones (e.g., PRL) and exerting vasoactive and immunomodulatory actions (3, 25, 26). Consequently, the wide spectrum of direct and indirect effects of PGs in the canine CL encouraged us to perform the present NGS-based study. The ultimate goal was to better understand the different roles that PGs might play in the CL transcriptome and, thereby, to elucidate other possible regulatory mechanisms induced by PGs in the CL. Taking advantage of access to control samples covering the time span between days 5 and 30 after ovulation, i.e., during the development and maturation of the CL, transcriptional changes were also assessed in these samples with regard to the effects of time. In agreement with our previous reports (3, 24, 26), large variations in gene expression were observed in the CL of Previcox-treated dogs. This may be at least partially explained by the small number of animals per group and individual variations in gene expression. However, as can be seen from the analysis of the sequencing data presented herein, treatment with Previcox itself seemed to be an additional cause for these fluctuations. In fact, samples from control groups showed a higher correlation with each other than samples from treated groups. This could also be observed on the heatmap analysis of the 2,000 genes with higher variance, showing more homogeneous clustering when only control samples were used. As discussed elsewhere, individual variations and pharmacokinetics may have played an important role in the lower homogeneity observed in treated groups (26). The evaluation of both PCA and heatmap plots also suggested a more homogeneous clustering of samples divided between early and mature CL than by application of treatment. It seemed,

TABLE 3 | Relative gene expression of target candidate genes affected by time in control animals.

Target	Group	RGE	SD+/SD–	ANOVA *P*-value	Dunn's test
TBXAS1	Day 5 control	3.95	1.57/1.12	0.0006	5C vs. 20C – $P < 0.05$ 10C vs. 20C – $P < 0.01$ 10C vs. 30C – $P < 0.05$
	Day 10 control	6.02	8.98/3.6		
	Day 20 control	1.75	0.86/0.58		
	Day 30 control	2.25	2.66/1.22		
PTGDS	Day 5 control	6.17	6.69/3.21	0.0153	10C vs. 20C – $P < 0.05$
	Day 10 control	15.02	18.43/8.28		
	Day 20 control	4.39	3.95/2.08		
	Day 30 control	6.74	14.58/4.61		
TGFβR1	Day 5 control	6.28	3.51/2.25	0.0017	5C vs. 30C – $P < 0.001$ 20C vs. 30C – $P < 0.05$
	Day 10 control	5.15	6.45/2.86		
	Day 20 control	6.49	4.86/2.78		
	Day 30 control	2.85	1.98/1.17		
ICAM1	Day 5 control	5.35	10.74/3.57	0.0003	5C vs. 20C – $P < 0.01$ 5C vs. 30C – $P < 0.01$ 10C vs. 20C – $P < 0.05$
	Day 10 control	3.23	1.76/1.14		
	Day 20 control	1.69	0.9/0.59		
	Day 30 control	1.83	0.77/0.54		
FAS	Day 5 control	2.61	1.01/0.73	0.0015	5C vs. 20C – $P < 0.01$ 5C vs. 30C – $P < 0.01$
	Day 10 control	2.33	2.43/1.19		
	Day 20 control	1.4	0.38/0.3		
	Day 30 control	1.45	0.77/0.5		
FASLG	Day 5 control	4.59	2.66/1.68	<0.0001	5C vs. 20C – $P < 0.01$ 5C vs. 30C – $P < 0.001$ 10C vs. 20C – $P < 0.05$ 10C vs. 30C – $P < 0.01$
	Day 10 control	4.2	2.05/1.38		
	Day 20 control	2.3	0.51/0.41		
	Day 30 control	1.85	1.13/0.7		
NFκB1	Day 5 control	1.63	0.63/0.46	0.0408	No significant effects in group comparisons
	Day 10 control	2.13	1.0/0.68		
	Day 20 control	1.35	0.72/0.47		
	Day 30 control	1.41	0.68/0.46		
NFκBIa	Day 5 control	2.4	3.85/1.48	0.0006	10C vs. 20C – $P < 0.001$
	Day 10 control	4.11	6.81/2.56		
	Day 20 control	1.2	0.33/0.26		
	Day 30 control	1.75	0.88/0.58		
FGF1	Day 5 control	3.2	1.57/1.05	0.0031	5C vs. 30C – $P < 0.01$
	Day 10 control	5.47	8.77/3.37		
	Day 20 control	4.98	2.05/1.45		
	Day 30 control	6.45	3.23/2.15		
FGF2	Day 5 control	3.63	2.04/1.31	0.0032	20C vs. 30C – $P < 0.01$
	Day 10 control	3.45	2.18/1.34		
	Day 20 control	4.65	1.46/1.11		
	Day 30 control	2.22	0.82/0.6		
THBS1	Day 5 control	8.91	5.75/3.5	<0.0001	5C vs. 20C – $P < 0.001$ 5C vs. 30C – $P < 0.001$
	Day 10 control	4.85	3.59/2.06		
	Day 20 control	1.97	1.4/0.82		
	Day 30 control	2.91	2.45/1.33		
PPARγ	Day 5 control	2.2	1.28/0.81	0.0183	10C vs. 20C – $P < 0.05$
	Day 10 control	4.08	6.75/2.54		
	Day 20 control	1.66	0.41/0.33		
	Day 30 control	1.89	0.9/0.61		
HSD17B7	Day 5 control	1.9	1.04/0.67	<0.0001	5C vs. 10C – $P < 0.05$ 5C vs. 20C – $P < 0.001$ 5C vs. 30C – $P < 0.001$
	Day 10 control	4.58	3.53/1.99		
	Day 20 control	7.7	1.74/1.42		
	Day 30 control	6.16	8.03/3.48		

Relative gene expression (RGE) is presented for each group as the geometric mean and geometric standard deviation (SD). Non-parametric ANOVA (Kruskal-Wallis) analysis was followed by Dunn's test. Only results considered statistically significant ($P < 0.05$) are presented. Blue ANOVA indicates higher gene expression in early control groups (days 5 and/or 10); red ANOVA indicates higher gene expression in mid-diestrus control groups (days 20 and/or 30); black ANOVA indicates no specific changing pattern between early and mid-diestrus control groups (i.e., between days 5 and/or 10 and days 20 and/or 30).

TABLE 4 | Relative gene expression of target candidate genes affected by treatment at each analyzed time-point.

Target gene	TaqMan real time PCR					NGS results	
	RGE/contr.	SD+/SD–	RGE/treat.	SD+/SD–	*P*-value	*P*-value	Log2 ratio
A. Day 5 treated over control							
TGFβ1	1.58	0.4/0.32	2.67	1.84/1.09	0.0017	0.02148	0.4632
FASLG	4.59	2.66/1.68	2.44	1.35/0.87	0.0013	0.03313	−1.4
FGF2	3.63	2.04/1.31	2.14	1.46/0.87	0.009	0.163	−1.136
THBS1	8.91	5.75/3.5	14.07	5.98/4.2	0.0129	0.2279	0.6146
SULT1E1	7.97	7.27/3.8	4.18	4.33/2.13	0.0023	0.003543	3.094
B. Day 20 treated over control							
TBXAS1	1.75	0.86/0.58	4.25	4.41/2.17	0.005	0.003262	2.014
PTGDS	4.39	3.95/2.08	28.31	28.06/14.09	<0.0001	7.43E-05	2.637
TGFβR1	6.49	4.86/2.78	2.18	1.91/1.02	0.0013	0.04251	−0.8656
ICAM1	1.69	0.9/0.59	16.76	23.31/9.75	<0.0001	4.21E-07	3.765
NODAL	4.5	11.64/3.24	262.07	227.7/121.8	<0.0001	2.98E-09	5.04
FAS	1.4	0.38/0.3	2.6	1.01/0.73	0.0003	0.006776	0.8808
FASLG	2.3	0.51/0.41	5.73	3.05/1.99	<0.0001	0.008149	2.836
NFκB1	1.35	0.72/0.47	2.42	1.04/0.73	0.0064	0.005073	0.932
NFκBIa	1.2	0.33/0.26	5.69	2.62/1.8	<0.0001	7.41E-10	2.01
FGF2	4.65	1.46/1.11	2.37	2.41/1.19	0.0162	0.4982	−0.6664
THBS1	1.97	1.4/0.82	12.77	10.58/5.79	<0.0001	0.0007533	2.55
PPARγ	1.66	0.41/0.33	5.56	7.24/3.14	0.0006	7.94E-08	2.283
NR4A1	4.96	3.03/1.88	43.73	44.88/22.15	<0.0001	2.11E-07	3.616
SULT1E1	6.62	6.63/3.31	17.15	32.76/11.25	0.0396	0.001586	2.63
C. Day 30 treated over control							
IDO1	4.75	6.1/2.67	1.63	0.97/0.61	0.0003	0.0002207	−3.172
FGF2	2.22	0.82/0.6	3.57	2.36/1.42	0.0088	0.765	−0.2163

Relative gene expression (RGE) is presented for each group (contr., control group; treat., treated group) as the geometric mean and geometric standard deviation (SD+/SD–). Student's t-test was applied to test the effect of treatment at each analyzed time-point. Only results considered statistically significant (P < 0.05) are presented. Blue t-test indicates higher gene expression in the control group, while red indicates higher gene expression in treated groups.

thus, that time had a great impact on the transcriptional changes observed among the samples studied.

Time-Dependent Effects

Showing homogenous distribution of gene expression, with a clear distinction between the early and mature CL, time-dependent effects were examined in control samples and additionally served for quality control. When compared with developing CL (days 5 and 10), more highly represented genes in the mature CL (days 20 and 30) related to lipid biosynthesis/metabolism. The canonical pathways predicted to be upregulated at this stage were related to cholesterol biosynthesis. Cholesterol is a substrate required for steroid hormone synthesis. An increase of its production is probably required for the observed increased steroidogenic output from the CL at this time. As also confirmed by the qPCR analysis, the expression of *HSD17B7* (known as PRLR-associated protein) was found to increase with maturation of the CL. Together with other isoforms of 17βHSD, this enzyme is responsible for the conversion of estrone into estradiol, a more potent estrogen (44, 45). Considering the high variation in circulating 17β-estradiol (E2) during the canine diestrus (1), together with the concomitantly increased expression of aromatase (CYP19) (46), it is plausible that the observed increase in *HSD17B7* expression could be involved in the local provision of estrogens in the canine CL. Reflecting the increasing steroidogenic capacity of the CL, following their initial post-ovulatory decrease, E2 levels increase during the course of diestrus in the dog, roughly following the P4 secretion profiles (13, 47). The expression of the estrogen receptors, ESR1/ERα and ESR2/ERβ, has been confirmed in the canine CL (46). Nevertheless, the involvement of estrogens in regulating canine CL function remains underexplored, even though some attempts were made to shed light on the underlying mechanisms (6, 46). Functional terms more highly represented on days 5 and 10 after ovulation were mainly related to immune function and proliferative mechanisms, such as locomotion, cell-cell adhesion, extracellular matrix organization, and regulation of the ERK1/2 cascade. Accordingly, pathways related to cell cycle, proliferation and immune function, were predicted to be inactivated following CL maturation, i.e., at days 20/30 of

the CL lifespan. The apparently increased immune activity in the developing canine CL is in accordance with its previously reported increased infiltration by macrophages, monocytes and lymphocytes at this stage (48, 49). Furthermore, regarding immune regulation, TNF was among the top predicted upstream regulators involved in the CL transcriptomic changes observed in response to time. Similarly, an increased expression of *TNFα* and its receptor *TNFR2* on day 5 compared with mature CL stages was reported previously (26). Here, the expression of *FAS* and *FASLG*, factors belonging to the TNF superfamily, was also found to be more highly expressed in early than in mature CL at mid-diestrus (days 5/10 over 20/30). Their increased expression at that time was corroborated by the qPCR results. The role of the FAS/FASLG system in luteolysis, as extrinsic inducers of apoptosis, has been widely described in different species (50–54). With regard to the early CL, FAS was also observed to be increased in bovine CL as early as at day 5 after ovulation, provoking the question of possible non-apoptotic FAS signaling in regulating CL function (55). Indeed, FAS has been shown to affect some downstream non-apoptotic signaling pathways, such as NFκB (56). Thus, although the role of FAS/FASLG in the developing CL is still unknown, it could be also related with immune-mediated tissue reorganization and proliferation. Similar to FAS/FASLG, thrombospondins (THBS) have been associated with luteolytic events, responding to PGF2α and acting as anti-angiogenic factors by inhibiting the pro-angiogenic FGF2 (57, 58). Similar to the rat (59), and as also found by our qPCR analysis, increased expression of *THBS1* was detected in early CL stages and decreased toward mid-diestrus in mature CL (days 20 and 30). Within the early CL stage, an intense angiogenic activity is observed. Thus, the increased expression of *THBS1* appears paradoxical and is not fully understood. It appears plausible that *THBS1* could act as a limiter of vascular overgrowth, as suggested by others (59). However, it should be mentioned that the protein availability of this factor, as well as the availability of its receptors, were not investigated in the present study, but certainly merit further attention.

Regarding eicosanoids, modulation of CL function is classically seen as a balance between the luteotropic function of PGE2 and the luteolytic activity of PGF2α. In the canine CL, the expression of PGE2 synthase (PTGES) and PGE2 receptor 2 (PTGER2/EP2) decreases with the passage of time (21). This expression pattern could explain inhibition of the eicosanoid signaling pathway predicted by IPA software. Accordingly, similar time-dependent effects were observed herein in the expression of *TBXAS1* and PGD2-synthase (*PTGDS)*, which decreased from the early to the mid-luteal phase in the transcriptional analysis (further confirmed by qPCR). Both eicosanoids have been predominanty characterized in other systems. Thus, whereas thromboxane 2 (TBXA2) has been related to platelet aggregation, myocardial ischemia and bronchoconstriction, PGD2 has been extensively described as a regulator of body temperature and sleep cycle, vasodilation, smooth muscles relaxation and bronchoconstriction (60–62). With regard to the reproductive systems, the inhibition of TBXAS leads to increased cAMP-dependent steroidogenesis in Leydig cells (63). As for PGD2, in males it acts as an activator of the Sox9 gene and is, therefore, pivotal in testicular organogenesis (64, 65). However, to the best of our knowledge, nothing is known about the modulatory effects these two eicosanoids might have on CL function. Strikingly, PGD2 can undergo spontaneous dehydration into different J prostanoids, such as 15d-PGJ2 (66, 67). This prostaglandin can mediate pro-inflammatory mechanisms through different pathways, but also affects anti-inflammatory responses, mainly through the nuclear receptor PPARγ (68). Expression of PPARγ decreased between days 10 and 20 in qPCR analysis, and is an alternative receptor for several different factors, including eicosanoids and fatty acids, with regulatory roles in fatty acid metabolism, cell differentiation and inflammation (69). Among other regulatory effects, PPARγ has an indirect role in potentiating the expression of STAR by upregulating cJUN (70). In the CL of pregnant dogs, it was stably expressed during the whole diestrus (39). However, here we observed increased expression of this receptor in early CL stages, similar to the two eicosanoid-synthases studied. Thus, it seems plausible that also in the dog PPARγ provides an alternative pathway for possible modulatory effects of PGD2-derived prostanoids in the CL.

Collectively, our analysis indicates that during the transition from the early developing to the mature CL a decrease in immune activity and tissue proliferation occurs, expectedly accompanied by its increased steroidogenic capacity. Additionally, among the predicted top upstream regulators, different hormones and PGE2 receptor 2 (PTGER2/EP2) were present, which are known to exhibit time-dependent changes in their expression in the CL during canine diestrus (14, 21). Taking into consideration that these functional changes and the expression of different genes were previously investigated and/or were expected, as mentioned elsewhere, the analysis of time-dependent effects served also as a primary validation of the Next Generation Sequencing methodology. Besides this, the expression patterns of *TBXAS*, *PTGDS*, *FAS/FASLG*, *NFκB/NFκBIA*, *THBS1*, *PPARγ*, and *HSD17B7* in the canine CL were described and discussed for the first time herein. All of these factors may serve functional roles in the development of CL in the canine species and thus constitute topics worthy of more attention in the future.

Treatment-Induced Effects

The functional suppression of COX2, and the consequent withdrawal of PGs, had variable effects on the different groups studied. The numbers of DEGs found for each studied time-point were also variable, being lower in gonadotropin-independent CL stages (days 5 and 10) than during the transition period and at gonadotropin dependency (days 20 and 30, respectively). Interestingly, the highest number of DEGs (1,741) was found in CL from dogs treated with Previcox for 20 days. This, together with the absence of genes commonly affected by treatment in all groups, reinforces the time and developmental stage-dependent effects of COX2 suppression on CL transcriptional activity. Furthermore, the presence of possible compensatory mechanisms for the withdrawal of PGs was suggested previously (3, 26) and appears to be a part of the inherent regulation of CL function in the dog. At the same time, the possible presence of such mechanisms advocates caution in the evaluation and

interpretation of the results obtained because they might be induced by these mechanisms, rather than being directly linked to the function of PGs.

Gonadotropin-Independent Stage of CL

This stage relates to the early, developing CL treated with Previcox over 5 and 10 days. Large individual and functional variations in the response to treatment were observed at this time at the transcriptome level, with fewer genes passing the applied stringent *P*-value and FDR criteria. Nevertheless, functional terms related to cellular movement and division dominated at day 5 after ovulation in response to Previcox. Additionally, the predicted activation of cell movement and cytoskeleton-related pathways was mainly due to the increased expression of factors like actins, laminin and myosin observed in the transcriptome analysis. In preceding studies, significant effects of treatment at this time-point showed decreased expression of STAR, PRLR and PTGES, the latter one being associated with lower levels of intra-CL PGE2 (3). However, no significant effects were observed regarding transcriptional capacity, vascularization or immune function (24, 26). Here, as detected by qPCR, expression of the growth factor *FGF2* was decreased by Previcox treatment, while *THBS1* was upregulated. Considering the aforementioned interaction between these factors, the expression pattern of *FGF2* and *THBS1* suggests a disruptive effect of this treatment on angiogenic mechanisms. The inhibitory effects on vascularization appear even more plausible when the increased expression of *TGFβ1* after treatment is considered. In fact, growth and capillary morphogenesis of endothelial cells isolated from bovine CL were diminished by this cytokine (71). Additionally, in the present study qPCR also detected decreased expression of *FASLG* in Previcox-treated CL. If, as described above, this factor is usually associated with anti-angiogenic activity, its increased expression in early CL stages could imply a positive role of *FASLG* in CL angiogenesis, which appears to be affected after treatment. Although hypothetical, this idea deserves further attention in the future.

Regarding other functional mechanisms, as indicated by qPCR, the gene expression of *SULT1E1* decreased after Previcox treatment. As indicated elsewhere, the auto/paracrine effects of estrogens in the canine CL have been proposed before (6, 46). SULT1E1 sulfoconjugates estrogens, disrupting their capacity to bind to their receptors and, in this way, preventing their actions on target tissues (72). Thus, the observed decrease of *SULT1E1* expression in response to treatment might disturb the balance of locally active estrogens in the CL, presumably as a part of the compensatory mechanisms following the withdrawal of PGs.

The effects of Previcox treatment on day 10 appeared less pronounced at the transcriptome level. In previous studies, however, we observed that inhibition of COX2 at day 10 decreased the expression of PTGES, consequently significantly decreasing the intra-CL levels of PGE2, and this was associated with a significant decrease in circulating P4 levels (3, 24). These previous findings apparently contrast with the low number of DEGs observed in the present analysis. It appears, however, plausible that the variation among the treated samples could be the culprit for the negative output from this analysis, in particular with regard to the applied adjusted *P*-value (FDR), accounting for multiple testing but not for the biological diversity in the response to the applied anti-COX2 insult.

Transition Toward Gonadotropin Dependence

This stage of the CL relates to the transitional period of development toward its gonadotropin dependence, represented in our study by day 20 after ovulation (17, 18). In fact, day 20 was the time-point most affected by treatment. At this time, the CL appears to be more susceptible to insults targeting its functionality. The main functional terms overrepresented in Previcox-treated samples at this day were related to cellular proliferation and immune response. This fits well with the previously reported reduced size of the nuclei of steroidogenic cells and increased expression of some pro-inflammatory interleukins (e.g., IL1β or IL6) in response to Previcox at this day (24, 26). In the present NGS analysis, IL1β and IL6 were also found to be differentially expressed after treatment. The activation of several immune system-related canonical pathways was predicted by IPA software, and increased expression of other pro-inflammatory factors, e.g., *ICAM1*, *NODAL*, *FAS*, *FASLG*, and *NFκB1,* was further confirmed by qPCR. This apparently increased reactivity of the immune system was accompanied by negative effects induced by the treatment at this time-point on the steroidogenic machinery, mirrored in the decreased expression of 3βHSD and STAR (3, 24). These findings suggest predominantly negative effects of treatment on CL function at this stage.

With regard to vascular function, in addition to the previously found increased expression of endothelin-1 (END1) and downregulation of angiopoietin 1 (ANGPT1) at day 20 of treatment (26), here, increased expression of the anti-angiogenic *THBS1* and decreased levels of *FGF2* were identified. With this, the postulated negative impact of PGs withdrawal on CL vascular activity was further substantiated.

Contrasting with the decreased expression of *SULT1E1* at day 5 of treatment, its expression was elevated during the transitional phase at day 20 in the CL of Previcox-treated dogs. SULT1E1 was also elevated in the CL in mid-pregnant dogs undergoing luteolysis after treatment with an antigestagen (27), suggesting that the local withdrawal of estrogens could be related with decreased CL activity. The apparently suppressed expression of *HSD17B7* ($P = 0.0608$) at day 20 of treatment further strengthens the idea of possible involvement of estrogens in CL function. On the other hand, we also observed strongly increased expression of the nuclear factor *NR4A1* (also referred to as Nur77) in response to Previcox. This expression pattern was further confirmed by qPCR. Several functions were previously described for this receptor. The expression of NR4A1 was increased in the CL of bitches, cows and rats in response to treatment with the luteolysin PGF2α (73–75). This receptor is also known to be an important regulator of inflammation [reviewed in (76)]. Thus, it is plausible that the increased expression of this nuclear receptor might be related to the increased inflammatory response observed at this CL stage to the Previcox insult. Nevertheless, its exact functions in the canine CL remain to be determined. Due to the versatile effects of NR4A1 in different organs and systems, its possible actions on the CL appear worthy of more attention in the future.

The increased expression of *TBXAS*, *PTGDS*, and *PPARγ* observed in transcriptomic and qPCR analyses at day 20 after Previcox treatment could represent possible compensatory mechanisms, as suggested in previous reports (3, 26). As mentioned elsewhere, PPARγ acts as an alternative receptor for prostaglandins (68, 69). Besides its potential to upregulate the pro-steroidogenic cJUN (70), it was shown to repress the activity of NFκB (77, 78). Indeed, increased expression of cJUN was proposed previously to counteract the negative effects of Previcox (26).

Cumulatively, in accordance with our previous findings (26), the functional inhibition of COX2, besides suppressing intra-CL PGE2 content (3), led to activation of CL immune system-related factors and pathways. It also negatively affected vascularization of the CL during its functional transition to the gonadotropin-dependent stage. The regulatory effects upon PPARγ and NFκB, the reciprocal interactions between FGF and THBS or even possible modulatory effects on locally produced estrogens, could possibly be involved in the maintenance of CL tissue homeostasis at this time in a PG-dependent manner.

Gonadotropin-Dependent CL Stage

In this comparison, the stage of CL development refers to mid-diestrus, represented by day 30. During this time, the maintenance of CL function is primarily dependent on PRL (19, 20). Day 30 showed the second highest number of DEGs in response to Previcox treatment. Also, in the Venn diagram analysis, days 20 and 30 shared the highest number of simultaneously affected genes, even if signaling pathways represented by these genes indicated their different functional status.

At day 30, the fully mature CL exhibits high steroidogenic capacity. This was reflected in the functional terms overrepresented at day 30 in control samples compared with their Previcox-treated counterparts. These included, e.g., steroidogenic and lipid metabolic processes, which were less represented in the CL of treated dogs. The analysis of functional pathways corroborated these observations, revealing cholesterol synthesis and steroidogenesis among the prevalent pathways affected by the treatment. Interestingly, in this contrast, the effects of the treatment appeared diverse and affected genes with higher functional variations. On the other hand, as indicated above, contrasting with day 20 of treatment, the immune system-related functional pathways (e.g., NFκB- and TGFβ-signaling) appeared less represented at day 30 in the treated CL. Their importance was, however, underlined by placing their respective associated factors among the top upstream regulators. Thus, besides TNF and PPARγ, TGFβ1 and NFκBIA (NFκB-associated factor) were also identified by IPA software. By adding new information, these results also fit well with the previously reported increased presence of CD4-expressing macrophages infiltrating the CL in response to treatment with Previcox at day 30 of treatment (26). Activation of the immune system was further indicated in the data set presented here by the strong suppression of IDO1 expression at day 30 of treatment. Being a rate-limiting enzyme in tryptophan catabolism, IDO1 function is considered to be a checkpoint in the activation of leukocytes by exerting immunosuppressive actions (79). Interestingly, and as indicated above, despite the proximities in the development time, maturation stage and steroidogenic capacity of the CL between days 20 and 30 after ovulation, the effects evoked upon the immune system at these two time-points by Previcox appeared to diverge, further highlighting the time-dependent effects of PGs withdrawal in the canine CL.

Final Remarks

As shown in this and previous studies, treatment of dogs with Previcox affects multiple CL components and functions (3, 24, 26). In accordance with these observations, broad effects of COX2 inhibition were observed in the deep RNA-Seq analysis performed herein. These effects were clearly stage-dependent. Day 20, marking the transitional period toward gonadotropin-dependence, was the most affected by COX2-inhibition, identifying this period as the most sensitive stage of CL development to functional PGs withdrawal. It appears that at this stage the intrinsic regulatory mechanisms become unstable, while the luteotropic effects of PRL may be affected by treatment (3). This could also affect the strong compensatory mechanisms present in earlier stages, rendering the mature CL less capable of stabilizing its transcriptome in response to the insult caused by Previcox treatment. Indeed, it appears that the early CL is more resistant to PGs withdrawal, suggesting that this organ is intrinsically regulated and bears strong compensatory mechanisms. With maturation of the CL, its transcriptome becomes more sensitive to COX2 inhibition.

Mechanisms related to cellular proliferation, immune system and vascularization are undoubtedly involved in the proper development of the CL. Accordingly, here, deeper insights have been provided into the regulatory mechanisms underlying CL development, identifying several factors and pathways that could play roles in this process. Some of these, such as THBS1 and FAS/FASLG, are known for their involvement in the termination of CL function, but their role in CL formation is still not well-understood. Additionally, the modulatory effects of estrogens and PPARγ in the canine CL are still obscure and may play important luteotropic roles. Finally, the increased expression of *TBXAS* and *PTGDS* in early CL stages supports the idea that other PGs, besides PGE2, may play an important role in regulating and supporting the canine CL.

Apparently, by investigating transcriptomic effects, and being based on gene expression patterns, the information presented herein is not definitive and further functional and protein expression-related studies are needed to support these findings and hypotheses. Nevertheless, our analyses with Previcox-treated dogs clearly reveal broader regulatory roles linked to PGs in CL function, besides the luteotropic support of steroidogenesis by PGE2 or the luteolytic signaling of PGF2α. With this, the translational aspect of the present study in relation to other domestic animal species is obvious.

Our study falls into the clinical trials category and Previcox was used orally, as recommended by the manufacturer. This might have weakened effects on the target tissue due to its metabolism, even though 10 mg/kg of firocoxib, double the clinically recommended dosage, was used (in consultation with the manufacturer regarding safety). Despite causing disturbances in CL function, in none of the dogs was the luteal phase terminated. From the clinical point of view, this is important information because administration of Previcox appears to be safe for CL function and maintenance in non-pregnant, and presumably, also in pregnant dogs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI's Gene Expression Omnibus, Series accession number GSE130369.

ETHICS STATEMENT

Animal experiments were approved by the responsible ethics committee (permit 54/2008) of the University of Warmia and Mazury in Olsztyn, Poland.

AUTHOR CONTRIBUTIONS

MTP was involved in developing the concept of the present study, experimental design, generating data, analysis and interpretation of data, and drafting of the manuscript. FG was involved in knowledge transfer, and in the laboratory part of the project, as well as in critical discussion of data. HR contributed knowledge transfer, critical discussion and interpretation of data, and editing the manuscript. TJ was involved in design of the *in vivo* study and tissue collection, knowledge transfer, critical discussion of data, and revision of the manuscript. BH was involved in design of the *in vivo* study. BH and AB were involved in knowledge transfer, critical discussion of data, and revision of the manuscript. MPK designed and supervised the project, and was involved in interpretation of the data, and drafting and revision of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00715/full#supplementary-material

Supplemental Table 1 | List of differentially expressed genes (DEGs) found in the contrast "days 20+30 control over days 5+10 control." All genes listed passed the criteria of $P < 0.01$ and FDR < 0.1 (False Discovery Rate, adjusted *P*-value).

Supplemental Table 2 | List of differentially expressed genes (DEGs) found in the contrasts "day 5 treated over day 5 control," "day 10 treated over day 10 control," "day 20 treated over day 20 control," and "day 30 treated over day 30 control." All genes listed passed the criteria of $P < 0.01$ and FDR < 0.1 (False Discovery Rate, adjusted *P*-value).

Supplemental Table 3 | Lists of representative genes (up to 10) and statistical analysis details of particular GO terms, particular functional networks, representative canonical pathways and top upstream regulators.

Supplemental Table 4 | Complete list of genes presented in Venn diagram from the contrasts "day 5 treated over day 5 control," "day 20 treated over day 20 control," and "day 30 treated over day 30 control" and each intersection.

Supplemental Table 5 | Relative gene expression and statistical analysis of all target candidate genes investigated with regard to time-dependent and treatment-induced effects. Relative gene expression (RGE) is presented for each group as the geometric mean and geometric standard deviation. For time-dependent effects, non-parametric ANOVA (Kruskal-Wallis) analysis was used, followed by Dunn's test. For the assessment of effects of treatment, Student's *t*-test was applied at each time-point analyzed. $P < 0.05$ was considered significant.

Supplemental Image 1 | Initial explorative analysis of the sequencing dataset. Samples correlation matrix and heatmaps were obtained by using CountQC app provided in the SUSHI framework. In **(A,B)**, "K" and "Ctrl" relate to control samples while "P" and "Treat" relate to Previcox-treated samples. **(A)** Sample correlation matrix containing all samples submitted for RNA-Seq and considering all genes present. Samples 10/1 (10_K_1) and 30/1 (30_K_1) controls, and 10/2 (10_P_2) and 30/1 (30_P_1) treated, exhibited low correlation coefficients compared with other samples from the same group and were removed from further analysis. **(B)** Sample correlation matrix containing final list of samples used in the present analysis and considering all genes present. Control groups appear to have higher homogeneity than respective treated groups. **(C)** Heatmap of 2,000 genes with higher variance among all control samples. Gene ontologies (GOs) shown were obtained with Enrichr. Samples show apparent better clustering than in **(D)**, the heatmap of 2,000 genes with higher variance among all samples analyzed (control and treated).

Supplemental Image 2 | Volcano plots of differentially expressed genes (DEGs; FDR < 0.1, Log2Ratio > 0.5) affected by the passage of time (**A**: contrast "days 20+30 control over days 5+10 control") or induced by treatment (**B**: contrast "day 5 treated over day 5 control;" **C**: contrast "day 5 treated over day 5 control;" **D**: contrast "day 5 treated over day 5 control").

Supplemental Image 3 | Functional networks found in the upregulated differentially expressed genes (DEGs) from the contrast "days 20+30 control over days 5+10 control" and in the downregulated DEGs from the contrast "day 30 treated over day 30 control." Functional networks were obtained with the ClueGO application for Cytoscape. The functional terms overrepresented in each group are shown. Redundant or non-informative terms were removed and the networks obtained were manually rearranged. Number of mapped genes is indicated by the node size while significance of functional terms is denoted by node color (represented in legend at the right bottom corner). **(A)** Networks more highly represented on days 20 and 30 control (mature CL) were related to intracellular transport and lipid metabolism. **(B)** Networks more highly represented in CL samples from control animals on day 30 after ovulation were related to nitric oxide synthesis and angiogenesis.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5. Discussion

5.1. Effects of Previcox treatment in the early canine CL

(Tavares Pereira, et al. 2019a; Tavares Pereira, et al. 2019b)

The independence of the canine CL on hypophysial support during its development is indeed one of the most interesting species-specific peculiarities in this species (Okkens, et al. 1990, Okkens, et al. 1986). This unique regulatory characteristic suggests that other factors may be involved in regulating development and function of early canine CL. Among them, PGs appear to be major players, in particular PGE2 acting through its cAMP-mediating receptors, PTGER2 and PTGER4 (Kowalewski 2014). Until recently, our knowledge regarding PGs effects in the canine CL was limited to the cAMP-dependent activation of steroidogenesis by PGE2, and its modulatory effects observed *in vitro* on the expression of PRLR, PTGER2, ETB, ET2, ANGPT1 and ANGPT2 (Gram, et al. 2015b, Gram, et al. 2018, Kowalewski, et al. 2013, Kowalewski, et al. 2015). Some of these effects were confirmed in our *in vivo* studies, further indicating the effects of PGs in regulating CL sensitivity to PRL (Kowalewski, et al. 2015). Thus, based on these previously published results, the present PhD thesis aimed to further investigate effects of Previcox-mediated withdrawal of PGs on the physiology of the canine CL.

Several effects have been attributed to PGs in different organs and systems, including those related to the modulation of vascular function and immune response (Breyer, et al. 2001, Hata and Breyer 2004). Accordingly, the investigations presented in Manuscript 1 (Tavares Pereira, et al. 2019a) focused on changes in the luteal expression of a selected range of factors from both these groups.
Regarding angiogenic and/or vasoactive factors, among the most interesting findings were the suppressive effects of treatment on all studied factors from the ANGPT-system. These contrasted with the absence of effects on VEGFA expression. As previously mentioned, interactions between the ANGPT system and VEGF are indispensable for normal angiogenesis, in which the loosening of blood vessels by ANGPT2 is required for the VEGF-induced angiogenic sprouting (Felcht, et al. 2012, Hanahan 1997, Yancopoulos, et al. 2000). In this way, adding to the effects PGE2 exerts on ANGPT family members *in vitro* (Gram, et al. 2018), here, the involvement of PGs in the regulation of luteal vascular development and maintenance has been confirmed by applying an *in vivo* approach. Following this avenue, regarding ETs, in the presented PhD thesis we also observed increased availability of ET1 in response to PGs-withdrawal on day 20. This finding suggests a modulatory capability of PGs upon the luteal blood supply mediated through the ET system.

Contrasting with CL formation and luteolysis, a lower presence of immune factors is observed in the mature CL (Hoffmann, et al. 2004a, Nowaczyk, et al. 2017, Zatta, et al. 2017). In this context, it was interesting to observe that the inhibition of COX2 in the CL resulted in enhanced expression of CD4 at day 30 and potentiated the expression of pro-inflammatory interleukins -1β, -6 and -12 at day 20 of the luteal phase. Therefore, the study presented herein for the first time implies the immunomodulatory, presumably immunosuppressive, roles of PGs in the CL, clearly indicating translational value of our findings to the understanding of CL function in other mammals as well.

The vaso- and immuno-modulatory roles of PGs in the canine CL described in Manuscript 1 pointed towards new, as yet undescribed, luteal roles of these eicosanoids. This prompted us to obtain a deeper insight into underlying regulatory mechanisms by applying the transcriptomic approach. This was achieved by the deep RNA sequencing method, as presented in Manuscript 2 (Tavares Pereira, et al. 2019b). Differential expression analyses (pairwise comparisons) were performed by comparing treated with control samples at each time-point (days 5, 10, 20 and 30 p.o.). Several target candidate genes were selected for validation of data and their expression was assessed by RT-qPCR. Functionally, these genes involved factors relating to eicosanoid synthesis, immune system, growth factors, vascular regulators, nuclear receptors and steroid-related factors.
Interestingly, samples distribution/homogeneity (observed in a PCA plot) appeared to rely more on the developmental stage of the CL than treatment, as two main clusters of samples could be observed: samples from the early developing CL (days 5 and 10 p.o.) formed one cluster, whereas the other was formed by samples from the developed CL (days 20 and 30). Nevertheless, significant effects of treatment could still be observed. After performing pairwise comparisons, differentially-expressed genes (DEGs) were filtered with the following criteria of P-value < 0.01 and False Discovery Rate (FDR, adjusted P-value) < 0.1. Day 20 was the most affected time-point, exhibiting 1741 DEGs, followed by day 30 with 552 DEGs. Interestingly, small effects of treatment were observed in the early CL stages, where 72 DEG were found on day 5 and 2 DEG on day 10 p.o. Interestingly, no gene was found to be concomitantly affected at all time-points. Cumulatively, these observations account for the most important discoveries from our investigations, as they deeply elaborate the time and development related genomic effects of Previcox treatment in the canine CL. These results also corroborate findings described in Manuscript 1 where most of the significant effects were observed on days 20 or 30 after ovulation.

Below, the main effects evoked by Previcox on the luteal transcriptome at major developmental stages of the canine CL are discussed separately.

5.1.1. Gonadotropin-independent stage of the CL (days 5 and 10)

In previous studies, Previcox treatment affected PGE2 synthesis and the steroidogenic function of the canine CL during the early gonadotropin-independent stage (Janowski, et al. 2014, Kowalewski, et al. 2015). Interestingly, these diminishing effects seemed not to be strongly observed in the present NGS analysis at this stage. It appears, however, plausible that the high variation among treated samples observed at this time, and the differences in sensitivity between qPCR and NGS methods, together with the stringent criteria of P-Value and FDR applied in RNA-Seq analysis, might have led to low numbers of DEGs found in transcriptomic analysis for the early (days 5 and 10) CL. On the other hand, these weak transcriptomic effects of treatment also suggest a resilience of the gonadotropin-independent CL to PGs-withdrawal, implying a strong intrinsic regulation of CL function at this stage.

Nevertheless, among the interesting findings observed at day 5 was the increased expression of thrombospondin 1 (THBS1) and downregulation of fibroblast growth factor 2 (FGF2) in response to treatment. THBS1 is an anti-angiogenic and pro-apoptotic factor that, in the bovine CL, is associated to luteolytic events (Niswender, et al. 2000, Schams and Berisha 2004). THBS1 can also block the expression and function of the pro-angiogenic FGF2 in bovine luteal cells *in vitro* (Farberov and Meidan 2014, 2016, Gospodarowicz , et al. 1986, Maroni and Davis 2011). Thereby, the results presented in the PhD thesis at the transcriptomic level further reinforce the presence of negative effects of PGs withdrawal on luteal angiogenesis.

5.1.2. Transition towards gonadotropin-dependence of the CL (day 20)

Highly contrasting with the effects observed in earlier gonadotropin-independent stages of the CL, as mentioned above, the highest number of DEGs identified after functional COX2 inhibition was found at day 20 of treatment. This stage represents the transition between the independence from gonadotropins and the required support of PRL for CL activity. One of the most important findings at this stage was the predicted activation of canonical pathways linked to immune activity after treatment. This observation was further supported by the increased expression observed herein of several pro-inflammatory factors, such as ICAM1, NODAL, FAS, FASLG and NFκB1 (Tavares Pereira, et al. 2019b), as well as the increased expression of IL1β, IL6 and IL12a (Tavares Pereira, et al. 2019a). Thus, an increased genomic immune reaction was observed after withdrawal of PGs in the canine CL on day 20 of the luteal stage.

Also interesting was the observed decreased expression of the vascular stabilizer ANGPT1 and of the pro-angiogenic FGF2, accompanied by the increased expression of the anti-angiogenic THBS1 and ET1. The possible interaction between THBS1 and FGF2 was also implied above for day 5 of Previcox treatment. The increase of ET1 expression was previously associated with luteolysis in

several species, e.g., ruminants, rats and rabbits (Boiti, et al. 2007, Hinckley and Milvae 2001, Ko, et al. 2012, Milvae 2000), and it was upregulated after the induction of luteolysis in mid-pregnant bitches treated with aglepristone (Gram, et al. 2015b). Following this, Previcox treatment also appeared to affect the stabilization of luteal vascularization at this stage.
Cumulatively, immuno- and vaso-modulatory effects of treatment were observed at this stage (day 20 p.o.). Further effects described previously related, e.g., to downregulation of STAR, 3βHSD and PRLR after treatment (Janowski, et al. 2014, Kowalewski, et al. 2015). With this, the transition between gonadotropin-independent and -dependent stages appears to be the most sensitive stage for inhibition of COX2-mediated PG synthesis in the first half of diestrus.

Following the observed decreased expression of STAR and lower P4 circulating levels after COX2-inhibition (Janowski, et al. 2014, Kowalewski, et al. 2015), we also investigated possible PG-dependent effects on the expression of factors regulating STAR transcription. Accordingly, cJUN is a well-known and one of the strongest regulators of STAR transcription (Stocco 2001) and its decreased expression was observed, concomitantly with decreased STAR levels, in pregnant dogs during prepartum luteolysis (Zatta, et al. 2017). For this reason, it was perplexing to observe the upregulation of cJUN after Previcox treatment. Similarly, CCNA2, a cell cycle regulator, was upregulated at day 20, when the transcriptional activity of luteal cells appeared to be decreased (Janowski, et al. 2014). The expression patterns observed herein of cJUN and CCNA2 were interpreted as a part of the compensatory response to treatment. This, as well as the presence of other, still unknown, compensatory mechanisms might explain why, despite luteal function being affected, it was not terminated after treatment (Janowski, et al. 2014, Kowalewski, et al. 2015). The compensatory effect could also explain the high variation in gene expression levels observed after treatment. Nevertheless, as mentioned elsewhere, it should also be noted that individual variations, the small number of animals per group, or even the pharmacokinetics of Previcox affecting its ovarian availability, could account for these observed variations.

As discussed before, the modulation of CL function is frequently attributed to the balance between PGE2 and PGF2α. For this reason, it was interesting to observe an increased expression of TBXAS1 and PTGDS in response to COX2-inhibition in animals from day 20 p.o. To the best of our knowledge, this is the first time the presence of these synthases has been described in the canine CL. Although possible roles of these eicosanoids in the CL are still unknown, one of the PGD2 targets might be the nuclear receptor PPARγ (Fitzpatrick and Wynalda 1983, Herlong and Scott 2006, Kikawa, et al. 1984, Komar 2005), which was also observed to be upregulated at day 20 in treated animals. Being an alternative receptor for several factors, including eicosanoids, PPARγ can

modulate a plethora of cellular mechanisms including pro-inflammatory response, by acting on NFκB or steroidogenesis, by indirectly increasing STAR expression through cJUN-dependent activation of its transcriptional activity (Delerive, et al. 1999, Kim, et al. 2007, Komar 2005, Kowalewski, et al. 2009b). Considering these broad effects of PPARγ, it is possible that the increased expression of PPARγ might be related to the increased expression of cJUN and/or increased immune signalling observed at this stage, which is a topic worth further investigations.

We were also interested in investigating PGs-mediated regulation of CL sensitivity to steroids. However, no effects of treatments with Previcox were observed on the luteal expression of ERα/ESR1 and ERβ/ESR2 at any time-point studied (Tavares Pereira, et al. 2019a). Together with the previously reported lack of effects of treatment on PGR expression (Kowalewski et al. 2015), these results suggest that the withdrawal of PGs has no effect on CL sensitivity to steroids. Nevertheless, an interesting finding was the upregulation of SULT1E1 in treated animals on day 20. SULT1E1 disrupts (by sulfoconjugation) the capacity of estrogens to bind to their receptors and in this way modulates availability of estrogens in target tissues (Song 2001). This finding suggests that PGs may still influence luteal estrogenic signaling by regulating the local provision of estrogens through SULT1E1 activity, rather than by modulating the nuclear ERs.

5.1.3. Gonadotropin-dependent stage of the CL (day 30)

Day 30 represents the mid-diestrus CL that depends on gonadotropin support for its function and exhibits high P4 production. In the transcriptomics analysis, canonical pathways related to vascularization were predicted to be inactivated by treatment at this stage. This was in concordance with the observed decreased expression of ANGPT2, Tie1 and Tie2. Additionally, the predicted inhibition of immune pathways at this stage was accompanied by the increased presence of factors like TNF, PPARγ, TGFβ and NFκBIA among the predicted upstream regulators for the observed changes in the transcriptome profile. This highly contrasted with the upregulation of several pro-inflammatory factors on day 20. Concomitantly, pathways linked to lipid metabolic process and steroidogenesis were also predicted to be inhibited in animals that received Previcox at day 30. Considering the dependence of the CL on PRL at this stage, it is possible that the observed effects could be indirectly induced through downregulation of the PRLR, as previously suggested (Kowalewski, et al. 2015). Furthermore, by presenting the diverging results obtained between days 20 and 30, mainly regarding immunomodulatory effects, the results obtained from this PhD thesis further highlight the stage-dependent effects of PGs withdrawal in the canine CL.

In summary:

With the results presented herein, the initial hypotheses underlying the present PhD thesis could be successfully confirmed. Thus, indeed, PGs exert multidirectional biological effects on the CL of the domestic dog, going beyond their functions in cAMP-dependent regulation of STAR. There are strong compensatory effects present in the CL upon withdrawal of PGs.

In particular we were able to show:

1) Broader and potentially novel roles of PGs in the regulation of the canine CL.

2) Effects of COX2-inhibition in the canine CL being clearly stage-dependent:

- **a.** Only weak effects were observed in the early independent CL stage, with treatment apparently having negative effects in the vascular development already as early in the luteal phase as at day 5 after ovulation.
- **b.** The transition towards gonadotropin dependence (represented by day 20 in the present study) appeared to be the most sensitive stage to treatment. COX2 inhibition remarkably stimulated pro-inflammatory responses in the CL, besides affecting different vascular factors. Covertly, these effects suggested an (immuno)protective role of PGs in the CL at this stage.
- **c.** Multidirectional effects of PGs withdrawal were observed in the transition to gonadotropin dependence (day 30). Effects of treatment on immune signaling at day 30 highly contrasted with the ones observed on day 20. Treatment also had a negative effect in steroidogenic activity and vascularization at this stage.

3) Changes in the expression of members of the ANGPT-system, ET1, THBS1 and FGF2 indicate an indirect role of PGs in the regulation of angiogenic processes and vascular stabilization in the canine CL.

4) Additional factors and mechanisms possibly involved in the regulation of CL function were identified. Some hypotheses that might be worth pursuing involve the possible SULT1E1-dependent regulation of local supply of estrogens in the canine CL in response to PGs, the THBS1/FGF2 interaction during the vascularization process, and the PPARγ-dependent effects of PGD2 in steroidogenesis and immune system activity.

5.2. Time-related changes in canine CL activity during the first half of diestrus

(Tavares Pereira, et al. 2019a; Tavares Pereira, et al. 2019b)

During development of the present PhD work, the availability of samples from control animals provided a great opportunity to evaluate time-dependent changes in gene expression during the development and maturation of the CL. This analysis also allowed identification of additional

factors possibly involved in the maturation and maintenance of the CL, with the expression of several genes being evaluated for the first time in the non-pregnant canine CL. Those include, i.a., the ANGPT and ET systems, eNOS and iNOS, THBS1, FGF1 and -2, ICAM1, TBXAS1 and PTGDS, as well as selected transcription factors involved in steroidogenesis, like cJUN, YY1, SF1, GATA4 and GATA6.

Considering the homogeneity of samples observed in the transcriptome analysis, estimated by their clustering in the PCA plot, the following contrast was created to study time-dependent effects: "days 20+30 control vs. days 5+10 control". From this contrast, a total of 3484 DEGs were found. Interestingly, mirroring the expected biological activity of the CL, together with the progression of its development and maturation, canonical pathways related to the immune system and cellular proliferation were predicted to be deactivated. This is in accordance with the previously described invasion of immune cells and upregulation of pro-inflammatory factors during CL development (Hoffmann, et al. 2004a, Nowaczyk, et al. 2017). At the gene expression level, elevated amounts of pro-inflammatory factors like IL10, ICAM1, CD4, TGFβ1 and NFκBIA were initially found, decreasing with the maturation of the CL, i.e., towards days 20 and 30 p.o. In addition, the TNF system was among the predicted upstream regulators. In fact, the expression of TNFα and TNFR1 was higher on day 5 p.o., decreasing with CL maturation. Similarly, the expression of FAS and FASLG was higher in the developing CL, consistent with their similar expression patterns observed previously during CL formation in the cow (Duncan, et al. 2012). It has been concluded the FAS/FASLG might be involved in immune mechanisms and morphological remodeling of the developing CL of the dog. Furthermore, following the predicted deactivation of cellular proliferation, the expression of CCNA2 decreased between days 5 and 20 after ovulation.

In a different direction, pathways linked to cholesterol synthesis and steroidogenesis were predicted to be activated during CL maturation. This is in accordance with published data because the mature CL exhibits increased expression of STAR, accompanied by higher output of P4 (Concannon 2011, Concannon, et al. 1989, Hoffmann, et al. 1992, Kowalewski and Hoffmann 2008). Also, corresponding with the increasing levels of STAR, the expression of SF1 increased towards maturation of the CL.

Another interesting finding from this project was the higher expression of THBS1 on day 5 p.o., compared with days 20 and 30. Considering the anti-angiogenic properties of THBS1, the increased expression of this factor during CL development was surprising. The ongoing remodeling processes or limitation of vascular overgrowth suggested previously by others (Petrik, et al. 2002) might be explanatory. In addition, the increased expression of TBXAS and PTGDS in early CL stages also suggests a role of TBXA1 and PGD2 in the development of the canine CL.

Similar to previous descriptions (Gram, et al. 2015b), the expression of ET2 decreased during the formation of the canine CL in the present work. However, contrasting with the absence of time effects observed in pregnant bitches (Gram, et al. 2015b, Gram, et al. 2018), the expression of ECE1, ANGPT2, Tie1 and Tie2 increased between the developing and mature CL stages in the present project. It should be, however, mentioned that the time-points and intervals studied previously in samples from pregnant animals (p.o. days 8-12, 18-25, 35-40, and prepartum luteolysis) diverge from the ones studied here (days 0, 5, 10, 20 and 30), which might explain the differences observed for gene expression in both studies. Regardless, the results obtained herein suggest the increased luteal availability of vasoactive factors towards maturation of the CL. Exhibiting a similar tendency, ETB expression was the lowest on the day of ovulation, increasing thereafter, concomitantly with elevated transcript levels of the inducible nitric oxide synthase (iNOS) and of endothelial nitric oxide synthase (eNOS). Apparently, thus, as in other species, in the dog also the vasodilatory effects of ETB are accomplished by activation of the nitric oxide pathway (Schneider, et al. 2007, Yanagisawa, et al. 1988).

Finally, in accordance with a previous report (Papa and Hoffmann 2011), time-dependent changes in the expression of estrogen receptors were observed in the present work. Interestingly, the higher availability of ESR1 and ESR2 in the matured CL was followed by similar expression patterns of HSD17B7 observed by NGS analysis. The latter, i.e., HSD17B7, is involved in the estrone (E1) to estradiol (E2) conversion (Nokelainen, et al. 1998, Stocco, et al. 2007), suggesting its involvement in the local supply of E2 in the canine CL.

In summary:

1) The time-dependent changes in the transcriptomic profile relate predominantly to cellular proliferation, activation of the immune system and steroidogenic function, and support the current views about canine luteal physiology (Kowalewski 2014).

2) New factors possibly involved in underlying biological and molecular mechanisms have been indicated. Accordingly, the expression of several factors involved in CL vascularization, immune system activity, availability of estrogens and regulation of STAR promoter activity were for the first time investigated in the canine CL.

3) Based on the described expression of PTGDS and TBXAS1, the capacity of the canine CL to synthesize eicosanoids other than PGE2 was described for the first time in the present PhD work. Regardless, the role of these eicosanoids in CL physiology is still unknown.

4) Despite the role of estrogens in the CL of the dog still being obscure, the expression patterns of HSD17B7 suggest its involvement in the local availability of these steroids.

6. Further studies and outlook

6.1. Expression profiles of PTGDS, TBXAS1 and factors involved in estrogen signaling in the canine CL during diestrus and potential modulatory roles of PGE2

6.1.1. Rationale

In the present PhD work, the expression of several factors in the canine CL, e.g., TBXAS1, PTGDS, SULT1E1 and HSD17B7, was investigated for the first time. Furthermore, the CL responses to Previcox implied new regulatory mechanisms. One of the most interesting was the possible regulation of local provision of E2 in the CL by SULT1E1 and HSD17B7. However, their expression was investigated only in the first 30 days of pseudopregnancy (Tavares Pereira, et al. 2019b). Thus, we aimed to further explore the presence of these factors in the CL throughout diestrus in both pregnant and non-pregnant animals. Additionally, the expression of ESR1, ESR2, and of the steroids sulfatase (STS), involved in counter-acting the inhibitory effects of SULT1E1 on E2 (Cole, et al. 2010, Purohit, et al. 1998, Rizner 2016), was investigated.

Moreover, as mentioned elsewhere, the expression of TBXAS1 and PTGDS suggested modulatory roles of TXA2 and PGD2 in the canine CL. The possible interaction between PGD2 and PPARγ, as well as the upregulation of these factors in response to treatment with Previcox, led us to investigate the expression of TBXAS1, PTGDS and PPARγ throughout diestrus. Considering the effects PGs-withdrawal had *in vivo* on the expression of several of these factors, we also investigated possible PGE2-mediated effects on the expression of these factors *in vitro* in early canine luteal cells.

6.1.2. Materials and methods

Luteal tissue samples from pregnant animals used in the present study were derived from our previous works (Gram, et al. 2015b, Kowalewski, et al. 2009a). Additionally, samples from non-pregnant bitches where kindly provided by Prof. Dr. Paula Papa (University of São Paulo, São Paulo, Brazil). Briefly, bitches from mixed breeds were monitored for the onset of spontaneous estrus by vaginal cytology and P4 assay. The day when P4 levels exceeded 5ng/ml was considered the day of ovulation. Non-pregnant samples (n = 5 animals/group) were then collected after 10, 20, 30, 40, 50 and 60 days. For pregnant samples, animals were mated 2 days after ovulation (day of mating was considered day 0 of pregnancy). Samples were then collected at the pre-implantation (days 8-12, n = 6), post-implantation (days 18-25, n = 5) and mid-gestation (days 35-40, n = 5) stages, and during pre-partum luteolysis (n = 4). The pre-implantation stage was confirmed by observing embryos in uterine flushes. To ensure the occurrence of pre-partum luteolysis, circulating

P4 levels were measured every 6h starting with day 58 of pregnancy until a continuous decrease in 3 consecutive measurements was observed. *Corpora lutea* were collected through OHE, dissected from surrounding tissues, placed in RNAlater for 24h at +4ºC and then stored at -80ºC until use.

Corpora lutea for luteal cell cultures were obtained from clinically healthy bitches (n = 3) in the early diestrus stage (days 14-21) that underwent routine OHE in the Department of Small Animals Reproduction, Vetsuisse Faculty, University of Zürich. Primary luteal cells were isolated and cultured as previously described (Kowalewski, et al. 2013) in 6-well plates with culture medium (DMEM/F12, pH 7.2-7.4, with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin, and 1% Insulin-Transferrin-Selenium). When 70-80% confluence was reached, the culture medium was replaced by serum-free medium (stimulation medium); cells were stimulated either with 1.0 mM N6,2-dibutyryladenosine-3,5-cyclic monophosphate (dbcAMP; positive control) or with 20 µM PGE2 for 6 h, which is the time period needed for maximal activation of STAR (Kowalewski, et al. 2013). Cells were collected with TRIzol and kept at -80ºC until RNA isolation. The expression of STAR was investigated in cAMP- and PGE2-treated cells for positive control (*not shown* ((Kowalewski, et al. 2013)).

The isolation of total RNA with the TRIzol reagent was performed following the manufacturer's protocol. The concentration and purity of RNA were measured with NanoDrop 2000c spectrophotometer. DNAse treatment for removal of possible DNA contamination and reverse transcription (RT) were done according to the previously published protocols (Kowalewski, et al. 2011, Kowalewski, et al. 2006). Finally, the total amount of cDNA was amplified using the TaqMan PreAmp Master Mix Kit and RT-qPCR and was performed as previously described (Tavares Pereira, et al. 2019a). All reactions were run in duplicate and negative controls were performed by replacing cDNA with autoclaved water and minus-RT control. Quantification of gene expression was performed by the ΔΔCt method (Kowalewski, et al. 2011, Kowalewski, et al. 2006), using the expression of three reference genes for data normalization (PTK2, KDM4A and EIF4H). Due to uneven distribution of RT-qPCR results, logarithmic transformation was performed and data are presented as geometric means (Xg) ± geometric standard deviation (SD). Evaluation of time-dependent effects was done by using the Kruskal-Wallis test (non-parametric ANOVA) followed by Dunn's test, while evaluation of cell culture results was performed with One-way parametric ANOVA followed by Dunnett's multiple comparison test, using controls as reference.

6.1.3. Main findings and physiological implications

All investigated target genes were found expressed in the studied samples.

Regarding eicosanoids synthases, no changes in the expression of TBXAS1 and PTGDS were observed during pregnancy (P>0.05, Fig. 3A and C). This strongly contrasted with their time-

dependent expression during non-pregnant diestrus ($P<0.0001$ for TBXAS1 and $P<0.0009$ for PTGDS, Fig. 3B and D). Thus, the expression of TBXAS1 was higher on day 40 than at any earlier time-point ($P<0.05$), and decreased subsequently towards day 60 ($P<0.05$, Fig. 3B). Also, PTGDS expression was significantly lower on day 30 when compared with the later stages of pseudopregnancy ($P<0.05$, Fig. 3D). In our previous study, the expression of PTGDS and TBXAS1 was highest on day 5 p.o., when compared with days 10, 20 and 30 (Tavares Pereira, et al. 2019b). Following this, the absence of day 5 in the present study may explain the lack of effects during luteal development. Nevertheless, the increased expression of these factors in the second half of diestrus, mainly at days 40 and 50, is quite interesting, as during this stage the expression of COX2 and of PTGES is decreased (Kowalewski, et al. 2008a, Kowalewski, et al. 2006). Adding this to the upregulation of these factors after the inhibition of COX2 on day 20 (Tavares Pereira, et al. 2019b), it appears that the expression of TBXAS1 and PTGDS might be increased in response to the decreased availability of COX2. Furthermore, similar to previous descriptions in pregnant bitches (Kowalewski, et al. 2011), no significant changes in the expression of PPARγ were observed in both pregnant and non-pregnant samples ($P>0.05$, Fig. 3E and F).

Regarding sensitivity to estrogens, stage-dependent effects in the expression of ESR1 (ERα) in pregnant animals were noted ($P<0.0001$), being lower ($P<0.001$) during pre-implantation than at mid-gestation (Fig. 4A). However, no significant effects in the expression of this factor were observed in pseudopregnant animals ($P>0.05$, Fig. 4B). Also, ESR2 (ERβ) remained stably expressed in both conditions ($P>0.05$, Fig. 4C and D). Considering the frequently opposing effects exerted by these receptors, as observed, e.g., in the mouse uterus where ERα promoted cellular proliferation while ERβ inhibited it (Weihua, et al. 2000), we further evaluated the expression ratio between ESR1 and ESR2. Interestingly, despite the high variation, this ratio decreased from early- (pre-implantation) to mid-gestation ($P< 0.01$), but increased significantly at prepartum luteolysis ($P< 0.05$, Fig. 4E). The receptor ratio was also higher in the slowly regressing CL on days 40 and 50 compared with the developing CL at day 10 ($P<0.05$ and $P<0.01$ respectively, Fig. 4F). Although effects of estrogens in the canine CL still need to be investigated, the possible involvement of these receptors in the regulation of cellular proliferation could explain the variations in the ESR1:ESR2 ratio observed herein. It needs, however, to be mentioned that the extent to which the data obtained at the transcript level would transfer to the ERα and ERβ protein levels remains to be investigated.

The expression of SULT1E1 increased from pre-implantation to mid-gestation ($P<0.01$), i.e., from approximately days 10-12 to 30-45 of pregnancy. It decreased thereafter, during prepartum luteolysis ($P<0.01$, Fig. 5A). A similar expression pattern was observed in non-pregnant dogs, in which SULT1E1 levels increased significantly between days 20 and 50 ($P<0.05$, Fig. 5B).

However, no decrease was observed during late regression of the CL, at day 60. Instead, SULT1E1 remained highly expressed at day 60 compared with day 30 ($P<0.05$, Fig. 5B). Due to its mentioned opposite activity to SULT1E1, the expression of STS was also investigated in the present study. In the CL of pregnant animals, STS appeared to present an opposite expression pattern to SULT1E1: it was abundantly expressed from pre-implantation to mid-gestation, but its representation was significantly lower in luteolytic CL compared with the pre-implantation stage ($P<0.05$, Fig. 5C). However, despite being continuously detectable in the CL of non-pregnant animals, STS did not differ significantly throughout diestrus in non-pregnant animals ($P>0.05$, Fig. 5D).

Also, the basic availability of HSD17B7 varied throughout diestrus ($P<0.0001$ in pregnant and $P<0.0003$ in non-pregnant animals, Fig. 5E and F). Its expression was higher during pre-implantation ($P<0.01$) and post-implantation ($P<0.001$) than in later stages of pregnancy (Fig. 5E). The first drop in its expression was noticed at mid-gestation ($P<0.05$), and it remained low thereafter, until prepartum luteolysis. In non-pregnant bitches, following the initial gradual increase towards day 40 after ovulation, the expression of HSD17B7 was suppressed ($p<0.05$) at day 50 (Fig. 5F).

As for the luteal cells, we found modulatory effects of PGE2 on ESR2 (ERβ) and PPARγ (Fig. 6A and B). Whereas ESR2 was positively affected ($P<0.05$), the expression of PPARγ was significantly suppressed ($P<0.01$). The expression of other factors was not affected by the treatment with PGE2 ($P>0.05$). In Manuscript 1 presented in this thesis (Tavares Pereira, et al. 2019a), the responsiveness of the CL to estrogens appeared to be unaffected by the withdrawal of PGs *in vivo*, contrasting with the results obtained here *in vitro*. These diverging effects might be caused by the high individual variation observed in the *in vivo* study. With regard to the diminishing effects of PGE2 on PPARγ expression, this negative response further suggests that the previously observed increase of PPARγ in COX2-inhibited samples (Tavares Pereira, et al. 2019b) might be due to the decrease of PGE2 within the CL (Kowalewski, et al. 2015). Considering that PPARγ was previously shown to have indirect effects on the expression of STAR (Kowalewski, et al. 2009b), these observations further support the role of PPARγ in a compensatory mechanism to the decreased PGE2-dependent steroidogenic activity of the canine CL.

It should be mentioned that while PPARγ mRNA levels remained unaffected, lower protein levels were observed during prepartum luteolysis in a previous study (Kowalewski, et al. 2011). For this reason, evaluation of protein levels appears required to confirm the effects presented herein . In fact, the investigation of protein levels and localization of some of the factors analyzed in this work is planned and will allow a deeper and more complete evaluation of their role in the canine CL. Nevertheless, the presented (unpublished) results suggest the involvement of PGD2, TBXA1, PPARγ and estrogens in the regulation of CL physiology in the dog.

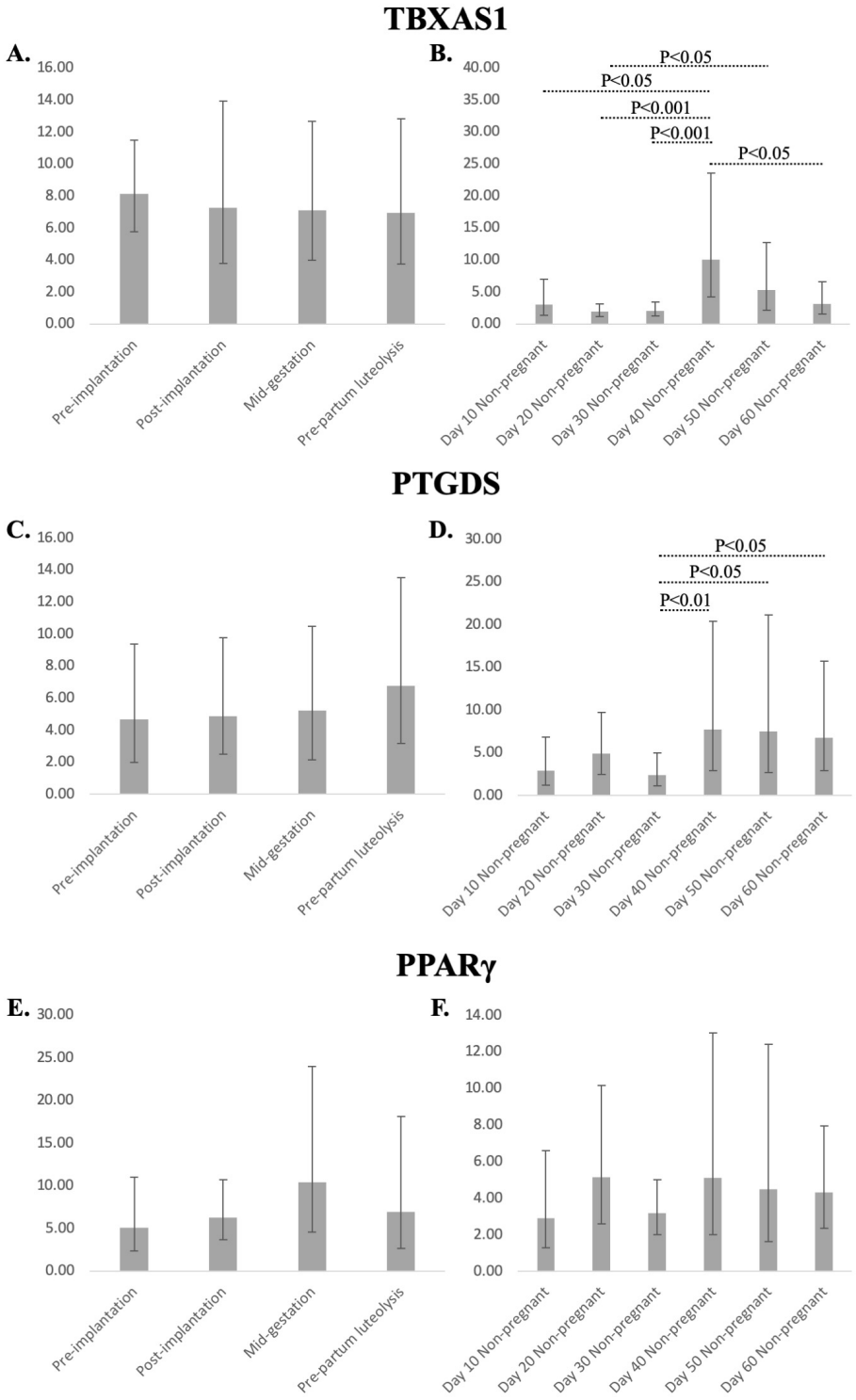

Figure 3. Relative gene expression of TBXAS1, PTGDS and PPARγ in the canine CL during pregnancy (A, C, E) and in non-pregnant diestrus (B, D, F) as determined by RT-qPCR (Xg ± SD). The sample with the lowest expression of each target gene was used as calibrator. Kruskal-Wallis (non-parametric ANOVA) was performed to evaluate time-dependent changes in gene expression during diestrus and revealed: (A) $P = 0.9229$, (B) $P < 0.0001$, (C) $P = 0.7734$, (D) $P = 0.0009$, (E) $P = 0.1103$, (F) $P = 0.2608$. When $P < 0.05$, Dunn's multiple comparison was performed and results are presented in the graphics (dotted lines).

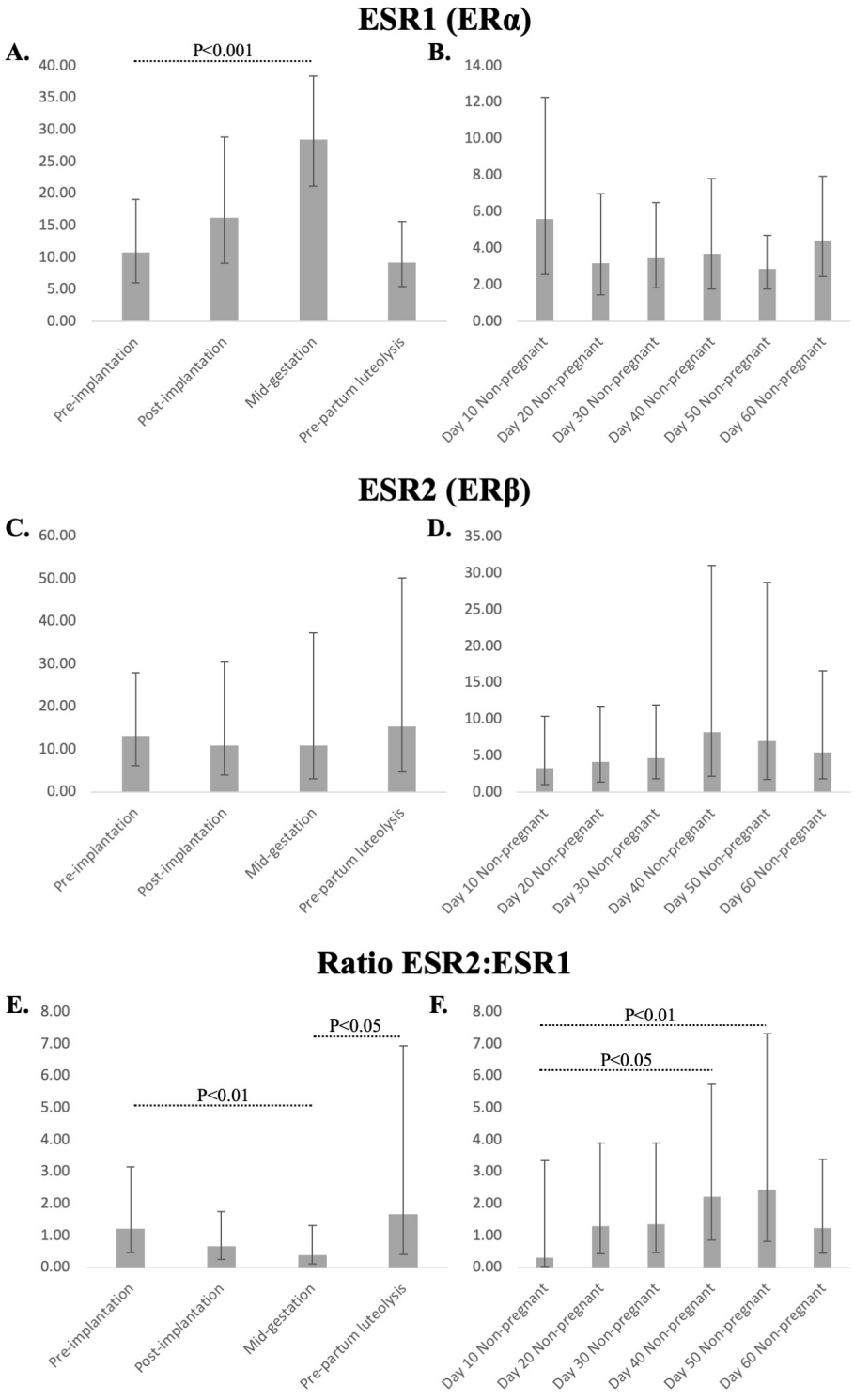

Figure 4. Relative gene expression of ESR1 (encoding for ERα), ESR2 (encoding for ERβ), and ratio between ESR1 and ESR2 expression in the canine CL during pregnancy (A, C, E) and non-pregnant diestrus (B, D, F) as determined by RT-qPCR (Xg ± SD). The sample with the lowest expression of each target gene was used as calibrator. Kruskal-Wallis (non-parametric ANOVA) was performed to evaluate time-dependent changes in gene expression during diestrus and revealed: (A) $P < 0.0001$, (B) $P = 0.2395$, (C) $P = 0.8643$, (D) $P = 0.4541$, (E) $P= 0.002$, (F) $P= 0.0056$. When $P < 0.05$, Dunn's multiple comparison was performed and results are presented in the graphics (dotted lines).

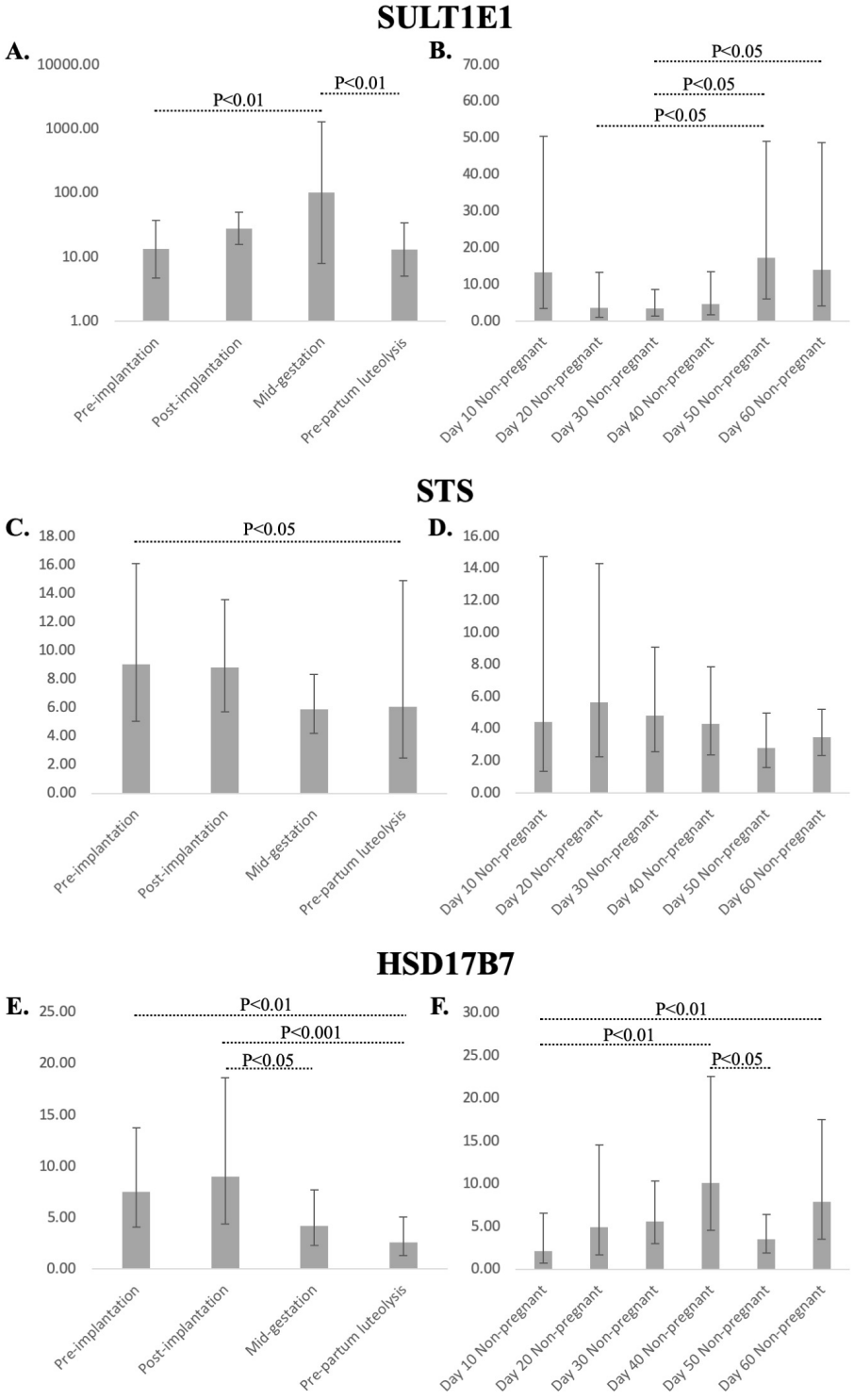

Figure 5. Relative gene expression of SULT1E1, STS and HSD17B7 in the canine CL during pregnancy (A, C, E) and non-pregnant diestrus (B, D, F) as determined by RT-qPCR (Xg ± SD). The sample with the lowest expression of each target gene was used as calibrator. Kruskal-Wallis (non-parametric ANOVA) was performed to evaluate time-dependent changes in gene expression during diestrus and revealed: (A) P = 0.0012, (B) P= 0.0002, (C) P = 0.0139, (D) P = 0.1958, (E) P < 0.0001, (F) P = 0.0003. When P < 0.05, Dunn's multiple comparison was performed and results are presented in the graphics (dotted lines).

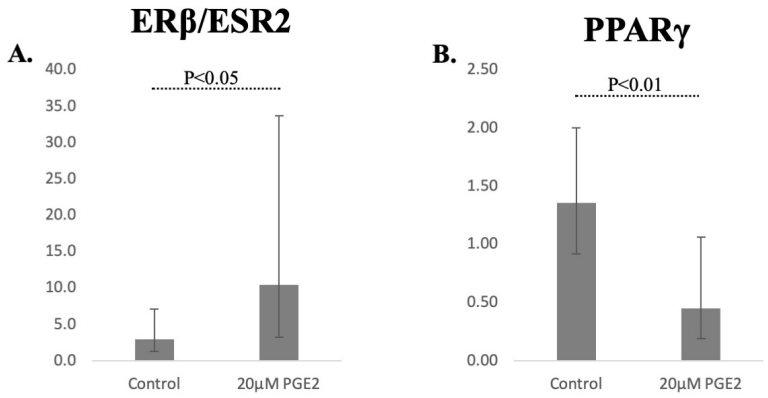

Figure 6. Relative gene expression of ESR2 (encoding for ERβ) and PPARγ in early primary canine luteal cells in the absence or presence of PGE2 as determined by RT-qPCR (Xg ± SD). Student's unpaired t-test was performed and revealed: (A) P = 0.0004 and (B) P = 0.0072.

6.2. Outlook

Despite the central role of the CL in canine reproduction, the regulatory mechanisms involved in its function and maintenance are still far from being fully understood. The stage-dependent differences in gene expression and response to treatment described in the present PhD work further highlight the complex mechanisms present in the regulation of canine CL function. In these regards, cell cultures, as applied in our still ongoing additional studies, are a useful tool to investigate effects in individual cellular populations, but lack the flexibility to explore cellular interactions. Thus, the development of a different model for the CL, where this cellular interaction could be assessed, appears to be needed.

Regardless, we were able to further explore the regulation of the canine CL in the present PhD thesis, uncovering new aspects of PGs activity. Nevertheless, as indicated above, the regulation of the canine CL still needs to be better explored, and the clinically available tools to modulate the canine reproductive cycle are limited. Thus, further understanding of canine reproduction, with a special focus on CL function, is still required for the development of new strategies to modulate the canine reproductive cycle.

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8. *Curriculum Vitae*

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September 2003 – July 2006	**School education** Colégio de Albergaria, Albergaria-a-Velha, Portugal
July 2006	**Highest school-leaving qualification** National exams of 12th grade, Colégio de Albergaria, Albergaria-a-Velha, Portugal
September 2006 – February 2013	**Studies** Integrated Master in Veterinary Medicine, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal
September 28th 2013	**Veterinary medicine degree examination** Master thesis defense, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal
February 2017 – March 2020	**Preparation of the thesis** under the direction of Prof. Dr. Mariusz P. Kowalewski at the Institute of Veterinary Anatomy Vetsuisse Faculty University of Zurich Director *ad interim*: Prof. Dr. Mariusz P. Kowalewski
May 2013 – August 2016	Small animals practitioner, Vet4 Lda., Estarreja, Portugal

9. Presentation of research results

The results described herein were presented at the following conferences and seminars:

Presentations at international meetings

19-21.09.2019 "Effects of *in vivo* inhibition of PTGS2/COX2 on the early canine *corpus luteum* (CL) transcriptome profile"
Oral presentation
Annual ESDAR Conference, St. Petersburg, Russia

14.09.2019 "Transcriptomic insights into *in vivo* inhibition of PTGS2/COX2 in early dioestric dogs"
Oral presentation
31st Pregnancy, Parturition, Postpartum, Perinatology Meeting, Lodi, Italy

20-22.02.2019 "Genome-wide transcriptional effects in the early *corpus luteum* (CL) of the dog after *in vivo* inhibition of prostaglandin synthesis"
Poster presentation (poster award)
52. Jahrestagung Physiologie und Pathologie der Fortpflanzung, gleichzeitig 44. Veterinär-Humanmedizinische Gemeinschaftstagung, Göttingen, Germany

10-13.07.2018 "*In Vivo* investigations of prostaglandin-mediated effects in early canine *corpus luteum* (CL) - new insights into luteal vascularization and immunity"
Oral communication and poster presentation
51st Annual Meeting of the Society for the Study of Reproduction (SSR), New Orleans, Louisiana, USA

29.09.2017 "*In vivo* investigations on function of prostaglandins in regulating early canine luteal function - new insights from the Previcox project"
Oral presentation
Workshop on Gonadal Function, Gamete Interaction and Pregnancy (GGP), Faculty of Veterinary Medicine, Justus-Liebig-University, Giessen, Germany

Presentations at national meetings

31.01.2019 "Effects of *in vivo* inhibition of prostaglandin synthesis in the early *corpus luteum* (CL) of the dog – transcriptome analysis"
Poster flash presentation
GCB Symposium, University of Bern, Bern, Switzerland

01.02.2018 "Prostaglandin-mediated effects on vascularization and immunomodulation in early canine *corpus luteum* (CL)"
Poster presentation
GCB Symposium (2018), University of Bern, Bern, Switzerland

Internal presentation of research results

08.02.2019 "Role of prostaglandins in formation of the *corpus luteum* in the dog: effects on vascularization, immunoactive factors and global transcriptomic changes"
Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

16.02.2018 "Role of prostaglandins in the early luteal formation in the dog: effects on vascularization and immunoactive factors"
Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

21.10.2016 "Formation and maintenance of canine *corpus luteum*: prostaglandin-mediated effects"
Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

Collaborations in other research projects during PhD work also produced results presented in the following congress:

20-22.02.2019 "Cellular mechanisms of canine species-specific decidualization: *in vitro* effects on remodelling processes, and roles of PGE2 and progesterone"
Poster presentation (presented by A. Boos)
52. Jahrestagung Physiologie und Pathologie der Fortpflanzung, gleichzeitig 44. Veterinär-Humanmedizinische Gemeinschaftstagung, Göttingen, Germany

10. List of Publications

Results obtained during the course of the present PhD work were described in the following publications:

(1) "Prostaglandin-mediated effects in early canine corpus luteum: in vivo effects on vascular and immune factors"
Tavares Pereira M, Gram A, Nowaczyk R, Boos A, Hoffmann B, Janowski T, Kowalewski MP; Reproductive Biology; Feb 2019; 10:100–11. doi: 10.1016/j.repbio.2019.02.001

(2) "Global transcriptomic analysis of the canine *corpus luteum* (CL) during the first half of diestrus and changes induced by *in vivo* inhibition of prostaglandin synthase 2 (PTGS2/COX2)"
Tavares Pereira M, Graubner F, Rehrauer H, Janowski T, Hoffmann B, Boos A, Kowalewski MP; Frontiers in Endocrinology; Nov 2019; doi: 10.3389/fendo.2019.00715

Further collaborations during the development of the present project resulted in the following publications:

(3) "Luteal ANGPT-TIE system during selected stages of pregnancy, and normal and antigestagen-induced luteolysis in the dog"
Gram A, **Tavares Pereira M**, Boos A, Grazul-Bilska AT, Kowalewski MP.; Reproduction; 2018 Aug 2 pii: REP-18-0222. doi: 10.1530/REP-18-0222.

(4) "Canine decidualization *in vitro*: extracellular matrix modification, progesterone mediated effects and selective blocking of PGE2 receptors"
Graubner FR, **Tavares Pereira M**, Boos A, Kowalewski MP
(manuscript under revision)

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12. Declaration of originality

Last name, first name: Tavares Pereira, Miguel

Matriculation number: 16-741-878

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date
Zürich, 10.01.2020

Signature